APPROVAL SHEET

Title of Dissertation: Modulation of Long-Term Potentiation and Epileptiform Activity in the Rat Dentate Gyrus by the Group II Metabotropic Glutamate Receptor Subtype mGluR3

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Doctor of Philosophy Degree
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ABSTRACT

Title of Dissertation:	Modulation of Long-Term Potentiation and Epileptiform Activity in the Rat Dentate Gyrus by the Group II Metabotropic Glutamate Receptor Subtype mGluR3

Paul Milward Lea IV, M.S.
Doctor of Philosophy, 2000

Dissertation Directed by:	John M. Sarvey, Ph.D. Professor, Department of Pharmacology
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We are the first to report involvement of group II metabotropic glutamate receptors (mGluRs; specifically mGluR3) in modulation of epileptiform activity and long-term potentiation (LTP) in the hippocampal dentate gyrus. By stimulation and/or inhibition of group II mGluRs in rat hippocampal slices, we discovered that: (1) N-acetylaspartylglutamate (NAAG; 50 and 200 μM) blocked LTP of extracellular excitatory post-synaptic potentials (EPSPs) after high-frequency stimulation (100Hz; 2s) of the medial perforant path, (2) the beta-isomer of NAAG (β-NAAG) and ethyl glutamate (100 μM; group II mGluR antagonist) prevented this blockade, and (3) β-NAAG did not affect EPSPs recorded in a paired-pulse paradigm which argues against a presynaptic effect. These data are the first to indicate competitive effects between β-NAAG and NAAG on mGluR3 receptors. β-NAAG s effects were characterized using cultured cells. In cerebellar granule cells, we found that: (1) β-NAAG did not affect inositol phosphate production stimulated by mGluR group I agonists glutamate, L-CCG-I, and quisqualate, (2) β-NAAG reversed decreases in forskolin-stimulated cAMP caused

by the mGluR group II agonist DCG-IV, and (3) β-NAAG did not reverse decreases in forskolin-stimulated cAMP caused by the mGluR group III agonist L-AP4. These results ruled out group I and III mGluRs as effectors of β-NAAG. We used cells stably transfected with mGluR2 or mGluR3 to determine that β-NAAG blocked forskolin-stimulated cAMP responses to glutamate, NAAG, the nonspecific group I, II agonist trans-ACPD, and the group II agonist DCG-IV via mGluR3, but not mGluR2. We conclude that β-NAAG is a specific antagonist of mGluR3. We used NAAG and β-NAAG to investigate the mGluR3 receptor contribution to epileptiform activity induced in hippocampal granule cells (considered gating cells for the spread of neuronal activity) using a high-potassium, low-calcium perfusate [Schweitzer, Patrylo, Dudek. J.Neurophys 68:2016]. We found that: (1) burst frequency is decreased by ethyl glutamate and β-NAAG, and (2) NAAG, after induction of spontaneous bursting, has no effect on burst frequency. We conclude that: (1) group II mGluRs modulate epileptiform bursting of granule cells, (2) mGluR3 rather than mGluR2 mediates this modulation, and (3) specific antagonists to mGluR3 may have potential therapeutic effects.

Modulation of Long-Term Potentiation and Epileptiform Activity in the Rat

Dentate Gyrus by the Group II Metabotropic Glutamate Receptor Subtype mGluR3

By

Paul Milward Lea IV

Dissertation submitted to the Faculty of the Department of Physiology Graduate Program

of the Uniformed Services University of the Health Sciences in partial fulfillment of the

requirements for the degree of doctor of Philosophy 2000

DEDICATION

To my wife, for her patience, love and forgiveness for those nights when I said, I ll be home in a little bit , turned into hours later. To my daughter Phoenix, born this year on February 13th, for opening my eyes to the more important things in life. To my parents, Paul and Geri, who gave me a childhood full of rich experiences. To my older sister, Paula, who continues to make me proud to be her brother. To her daughter Bryn who teaches the act of kindness by showing how to give gifts from the heart. To Mrs. Carlig, my 7th and 8th grade science teacher who helped set me on this course in my life. To John Sarvey, a true mentor in every sense of the word. Finally, to my Grandparents Elda Lea, Paul Lea, Jr., Mary Guede and Albert Guede, who are the cornerstones of our family, I will always have a place in my heart and memory for all of you; I hope that I have made you all proud. I miss you Grandma Lea.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

4CPG	Group I mGluR antagonist
4-AP	K^+ channel blockers
DNQX	AMPA receptor antagonist
(1S,3R)-ACPD	Group I and II mGluRs agonist
ACPD	1-aminocyclopentane-1,3-dicarboxylic acid
ACSF	Artificial cerebral spinal fluid
ω-Agatoxin IVA	Blocker of P type Ca^{2+} channels
AHP	After hyperpolarization
AMPA	(S)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP4	L(+)-2-amino-4-phosphonobutyric acid
APB	2-amino-4-phosphonobutyrate
APV	DL-2-amino-5-phosphonovaleric acid
Barium	K^+ channel blockers
BHK	Baby Hamster Kidney cells
Bicuculline	$GABA_A$ receptor antagonist
bis-indolylmaleimide	Specific PKC inhibitor
calphostin C	Blocker of PKC-mediated phosphorylation
cAMP	Cyclic adenosine-3 ,5 -monophosphate
cDNA	Clonal dideoxynucleic acid
CGP-55845	$GABA_B$ receptor antagonist
charybdotoxin	K^+ channel blockers
chelerythrine chloride	PKC blocker
CHO	Chinese Hamster Ovary cells
CNQX	AMPA/kainate receptor antagonist
CNS	Central nervous system
AP-5	NMDA receptor antagonist
DAA	D-α-aminoadipate
DAG	Diacylglycerol
DCG-IV	2S,2 R,3 R)-2-(2 ,3 -dicarboxycyclopropyl)glycine
dendrotoxin,	K^+ channel blockers
EPSC	Excitatory postsynaptic current
EPSPs	Excitatory postsynaptic potentials
ethyl glutamate (EGlu)	Group II mGluR specific antagonist
GABA	γ-Aminobutyric acid
gc	Granule cell
GCPII	Glutamate carboxypeptidase II
GDEE	Glutamic acid diethyl ester
GDP-β-S	Inhibitor of G-proteins by keeping them in an inactivated

	state
genistein,	Protein tyrosine kinase inhibitor
GLAST	High-affinity glutamate transporter
glibenclamide	K^+ channel blockers
GTP-γ-S,	Irreversible activator of G-proteins
GTP.	Guanosine triphosphate
HFS	High-frequency stimulation
i1, i2, i3 and i4	The intracellular loops of mGluRs
I_{AHP}	Slow Ca^{2+}-dependent afterhyperpolarizing K^+ current
IBMX	Phosphodiesterase inhibitor
Ictal	Relating to or caused by a stroke or seizure
$I_{K(Ca, slow)}$	Slow Ca^{2+}-dependent current
$I_{K(slow)}$	Slow Ca^{2+}-independent current
$I_{K(leak)}$	Leak potassium current
I_M	Voltage-dependent slowly inactivating current
Interictal	The period between convulsions
IP_3	Inositol triphosphate
IPI	Interpulse intervals
KA	Kainic-acid
L-AP4	Group III mGluR agonist
lavendustin-A	Protein tyrosine kinase inhibitor
L-Glu	Glutamate
LPP	Lateral perforant path
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MCPG	Group I mGluR agonist
mGluR	Metabotropic glutamate receptor
MK801	NMDAR antagonist
MPP	Medial perforant path
MPP-gc or LPP-gc	Perforant path-granule cell synapses
mRNA	Messenger RNA
ms	Millisecond
NAA	N-acetyl-aspartate
NAAG	Endogenous N-acetyl-L-aspartyl-L-glutamate (α isomer)
NBQX	AMPA receptor antagonist
NMDA	N-methyl-D-aspartate
phorbol 12,13-diacetate	PKC activator
Phorbol esters	Activators of PKC
phorbol-12-13-dibutyrate	PKC activator

picrotoxin	GABA$_A$ receptor antagonist
PKA	Protein kinase A

PKCI	PKC inhibitory peptide ; blocker of PKC-mediated phosphorylation
PKC,	Protein kinase C
PKC19-31	PKC inhibitor
Postictal	Following a seizure
PTX	Pertussis Toxin
PTZ	Pentylenetetrazol
quisqualate	Group I and AMPAR agonist
QX-314	Na^+ channel blocker
RGS4,	A regulator protein of G-protein signaling
SCN	Suprachasmatic nucleus neurons
sphingosine	Blocker of PKC-mediated phosphorylation of the NMDA receptor
staurosporine	Blocker of PKC-mediated phosphorylation
TBA	Detects lipid hydroperoxides and lipid oxidation
TEA,	K^+ channel blockers
TM	Transmembrane
trans-ACPD	Trans-1-aminocyclopentane-1,3-dicarboxylate
trans-ACPD	Group I and II mGluRs agonist
tyrophostin-B42	Protein tyrosine kinase inhibitor
Walsh peptide	Inhibitor of cAMP-dependent protein kinase
β-NAAG	β-isomer of NAAG

Background

N-acetyl-L-aspartyl-L-glutamate (NAAG)

The endogenous neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) was

discovered in 1964 (Curatolo, 1964). In 1967, NAAG was identified in the human brain

(Auditore *et al.*, 1966). Since its discovery NAAG has been shown to be widely

distributed throughout the mammalian central nervous system (for review see (Coyle,

1997). In addition, NAAG has been shown to be synthesized in the CNS (Cangro *et al.*,

1987) and colocalized in glutamatergic (Tsai *et al.*, 1993b) and nonglutamatergic systems

(Forloni *et al.*, 1987). In various model systems, NAAG has been shown to be released

by a Ca^{2+}-dependent mechanism (Pittaluga *et al.*, 1988; Tsai *et al.*, 1988; Williamson and

Neale, 1988a; Zollinger *et al.*, 1988), absorbed *via* the specific transporter PEPT2 (Wang

et al., 1998), and/or hydrolyzed by glutamate carboxypeptidase II (GCPII, NAAG

dipeptidase) into N-acetyl-aspartate (NAA) and L-glutamate (Blakely *et al.*, 1986;

Robinson *et al.*, 1987; Williamson and Neale, 1992).

If taken together, these findings suggest that NAAG is a neurotransmitter.

Unfortunately, faulty methods used prior to 1988, and a somewhat confusing web of

terms describing the actions of NAAG at the N-methyl-D-aspartate (NMDA) receptor has

made an understanding of NAAG s function difficult.

One of the first papers to identify problems with earlier methods used to study

NAAG as a neurotransmitter was by Whittemore and Koerner (Whittemore and Koerner,

1989). They concluded that some of the NAAG-evoked depolarizations, reported earlier

(Ffrench-Mullen *et al.*, 1985), were confounded by extracellular potassium. Around the

same time, Blakely and colleagues (Blakely *et al.*, 1988) cautioned about interpretations

of ligand binding assays in which the ligand is subject to enzymatic modification. They

concluded that earlier interpretations of NAAG binding to a subclass of glutamate

binding sites (referred to as A4; nomenclature of (Foster, 1984)) were incorrect. To date,

much of the research on NAAG as a possible neurotransmitter prior to 1987 is no longer

considered applicable (Neale, personal communication).

The action of NAAG at the NMDA receptor is controversial. NAAG has been

reported as an agonist (Joels *et al.*, 1987; Mori-Okamoto *et al.*, 1987; Sekiguchi *et al.*,

1989; Sekiguchi *et al.*, 1992; Bos and Mirmiran, 1993; Puttfarcken *et al.*, 1993;

Valivullah *et al.*, 1994), and/or antagonist (Grunze *et al.*, 1996), and/or mixed

agonist/antagonist (Puttfarcken *et al.*, 1993). One possible explanation for these findings

is that NAAG is a partial agonist at the NMDA receptor. In Appendix A, I present why

by pharmacological definition NAAG should be reported as a partial agonist. Therefore,

throughout the remainder of this dissertation NAAG will be referred to as a partial

agonist at the NMDA receptor.

Despite the problems reported above, an understanding of what NAAG and

GCPII do in the CNS is important because many diseases and disease models show

alterations in NAAG and GCP II levels. A summary of neurodegenerative disease related

research shows that NAAG decreases in Huntington s disease (Passani *et al.*, 1997),

Alzheimer s disease (Jaarsma *et al.*, 1994; Passani *et al.*, 1997), decortication, kainate

lesioning of striatum and hippocampus, spinal cord transection (Koller *et al.*, 1984) and

ALS (Rothstein *et al.*, 1990; Tsai *et al.*, 1991; Plaitakis and Constantakakis, 1993; Tsai *et

al.*, 1993a). In addition, NAAG is increased and NAAG dipeptidase is decreased in

schizophrenic brains (Tsai *et al.*, 1995), kindling models of epilepsy (Meyerhoff *et al.*,

1985; Meyerhoff *et al.*, 1989; Meyerhoff *et al.*, 1992c), and in pentylenetetrazol (PTZ)-induced seizures (Brancati and D'Arcangelo, 1991). Perhaps the recent findings of Wroblewska and colleagues (Wroblewska *et al.*, 1997), who show that NAAG is a specific agonist at the metabotropic glutamate receptor subtype mGluR3, has brought us closer to understanding a role for NAAG in the CNS.

The Metabotropic Glutamate Receptor Family

Metabotropic glutamate receptors (mGluRs) were independently discovered in the 1980s by Weiss and Hirono as G-protein coupled receptors which bind glutamate and activate phosphoinositide hydrolysis (Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987), respectively). The presence of this new family of glutamate binding receptors was subsequently confirmed in the labs of Nakanishi and Hagen in 1991 with the independent deduction of mGluR cDNA amino acid sequences (Houamed *et al.*, 1991; Masu *et al.*, 1991), respectively). Since 1991, a total of eight separate mGluR sequences and a variety of splice variants have been identified.

Extracellularly, the mGluRs as a family have a large glutamate binding domain (O'Hara *et al.*, 1993); Figure 1) and 21 cysteine residues in conserved positions. These cysteine residues are believed to be responsible for dimerization (Romano *et al.*, 1996). However, it remains to be seen what if any role dimerization may have on functionality *in vivo* and/or *in vitro*. There are seven putative transmembrane (TM) domains forming four intracellular (i) loops. The proposed fourth intracellular loop (i4) is formed by palmitoylation of 15-25 cysteine residues downstream of the seventh TM domain (Pin,

Figure 1. The metabotropic glutamate receptor. A schematic showing the large

extracellular glutamate binding domain and the extracellular cysteine residues in

conserved positions. There are seven putative transmembrane (TM) domains forming

four intracellular (i) loops. The proposed fourth intracellular loop (i4) is formed by

palmitoylation of 15-25 cysteine residues downstream of the seventh TM domain.

Intracellular loops i2, i3 as well as a small section of i4 have been shown to be regulators

of G-protein binding. This figure was modified from Conn and Pinn, 1997.

Glu-binding domain

Cys-rich domain

7 Transmembrane domain

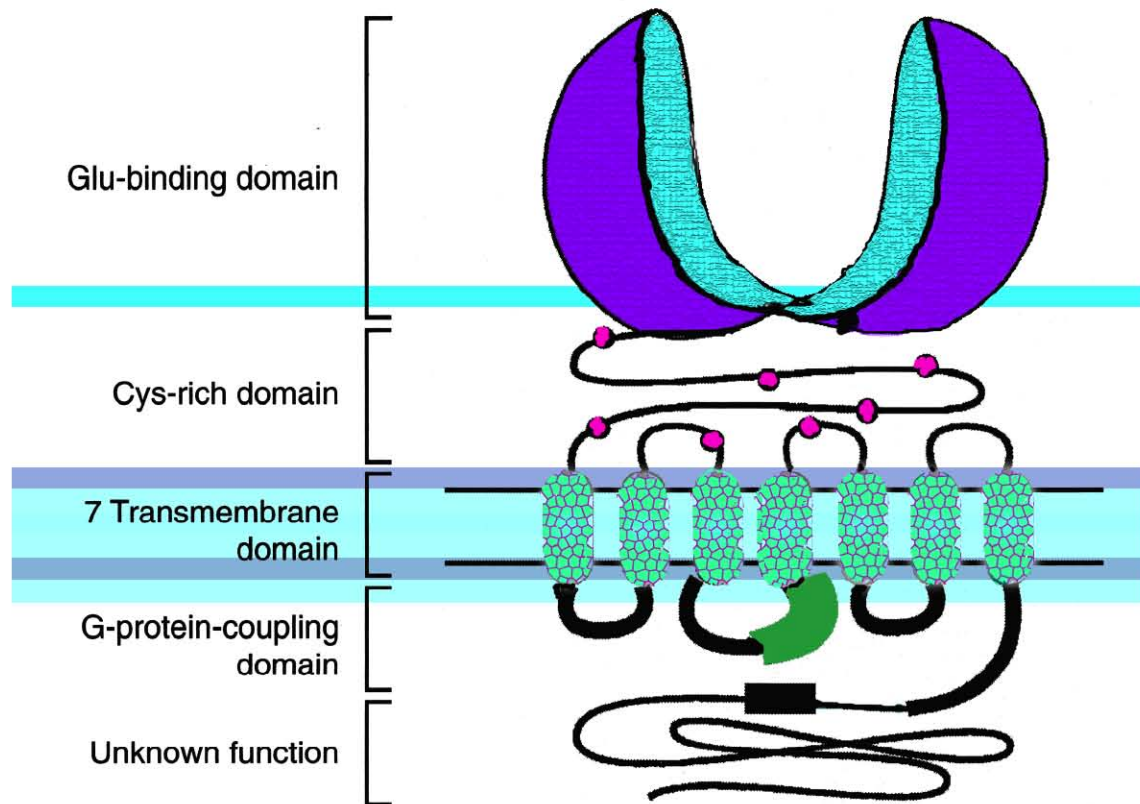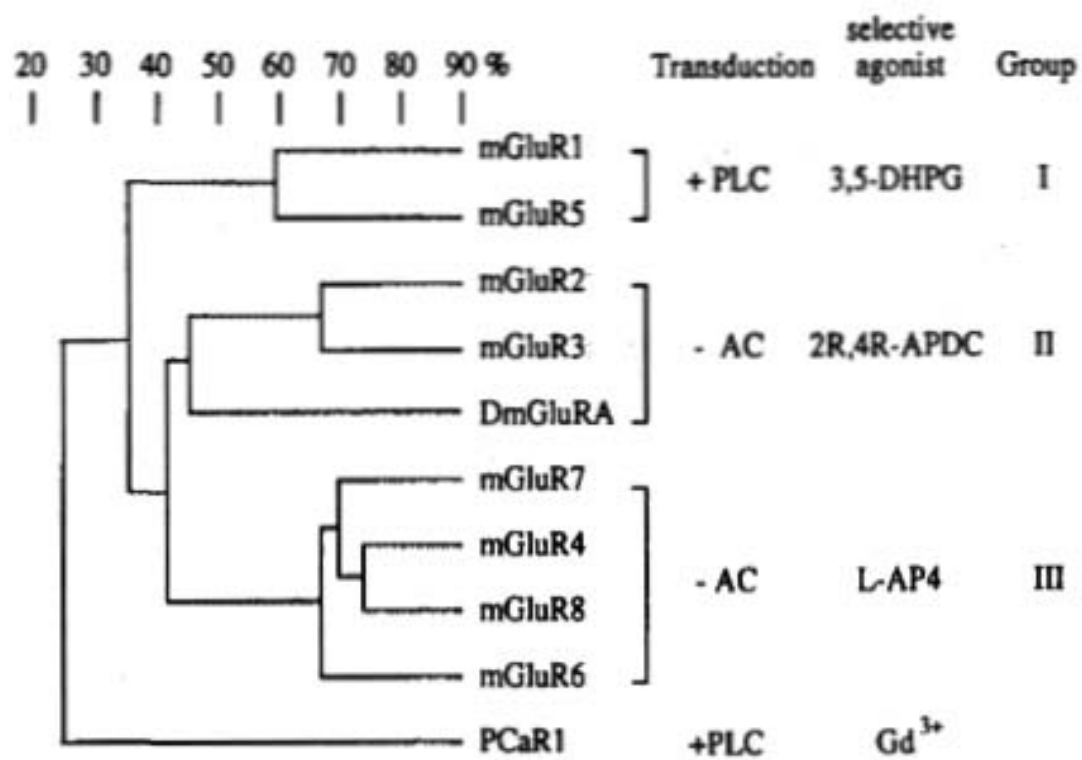G-protein-coupling domain

Unknown function

Figure 2. The organization of the mGluR family. This figure shows the three groups of mGluRs based upon sequence, pharmacology, and function. The within-group sequence homology is approximately 70%, whereas between groups it is approximately 45%. The Group I mGluRs consist of the mGluR1 and mGluR5 subtypes. They activate phospholipase C *via* G_q proteins and initiate an IP3/DAG second messenger cascade. The Group II mGluRs consist of the mGluR2 and mGluR3 subtypes. They inhibit adenylyl cyclase *via* $G_{i/o}$ proteins and block the cAMP second messenger cascade. The Group III mGluRs consist of the mGluR4, mGluR6, mGluR7 and mGluR8 subtypes. They also inhibit adenylyl cyclase *via* $G_{i/o}$ proteins and block the cAMP second messenger cascade. This figure was taken from Conn and Pinn, 1997.

1998). Intracellular loops i2, i3 as well as a small section of i4 have been shown to be regulators of G-protein binding (Pin, 1998).

The mGluRs are categorized into three groups based upon sequence, pharmacology, and function (see (Conn and Pin, 1997; Anwyl, 1999; Schoepp *et al.*, 1999) for reviews). The within-group sequence homology is approximately 70%, whereas between groups it is approximately 45% (Figure 2).

The Group I mGluRs consist of the mGluR1 and mGluR5 subtypes. They activate phospholipase C *via* G_q proteins and initiate an IP3/DAG second messenger cascade. The Group II mGluRs consist of the mGluR2 and mGluR3 subtypes. They inhibit adenylylcyclase *via* $G_{i/o}$ proteins and block the cAMP second messenger cascade. The Group III mGluRs consist of the mGluR4, mGluR6, mGluR7 and mGluR8 subtypes. They also inhibit adenylylcyclase *via* $G_{i/o}$ proteins and block the cAMP second messenger cascade. Currently it is believed that group I mGluRs are located both pre-and postsynaptic, group II mGluRs are located presynaptically (mGluR2 and 3) and on glial cell membranes (mGluR3), and group III mGluRs are both pre-and postsynaptic (Conn and Pin, 1997).

Although, the general absence of specific pharmacological agents has hindered the identification of specific mGluR subtype effects on neuronal and glial cells, various groups have deduced effects through a myriad of experimental designs and models. In general, the mGluRs have been shown to be modulators of various calcium (Sayer *et al.*, 1992; Swartz and Bean, 1992; Sahara and Westbrook, 1993; Swartz *et al.*, 1993; Chavis *et al.*, 1994; Rothe *et al.*, 1994; Stefani *et al.*, 1994; Chavis *et al.*, 1995a; Chavis *et al.*, 1995b; Chavis *et al.*, 1996; Choi and Lovinger, 1996; Stefani *et al.*, 1996a; Stefani *et al.*,
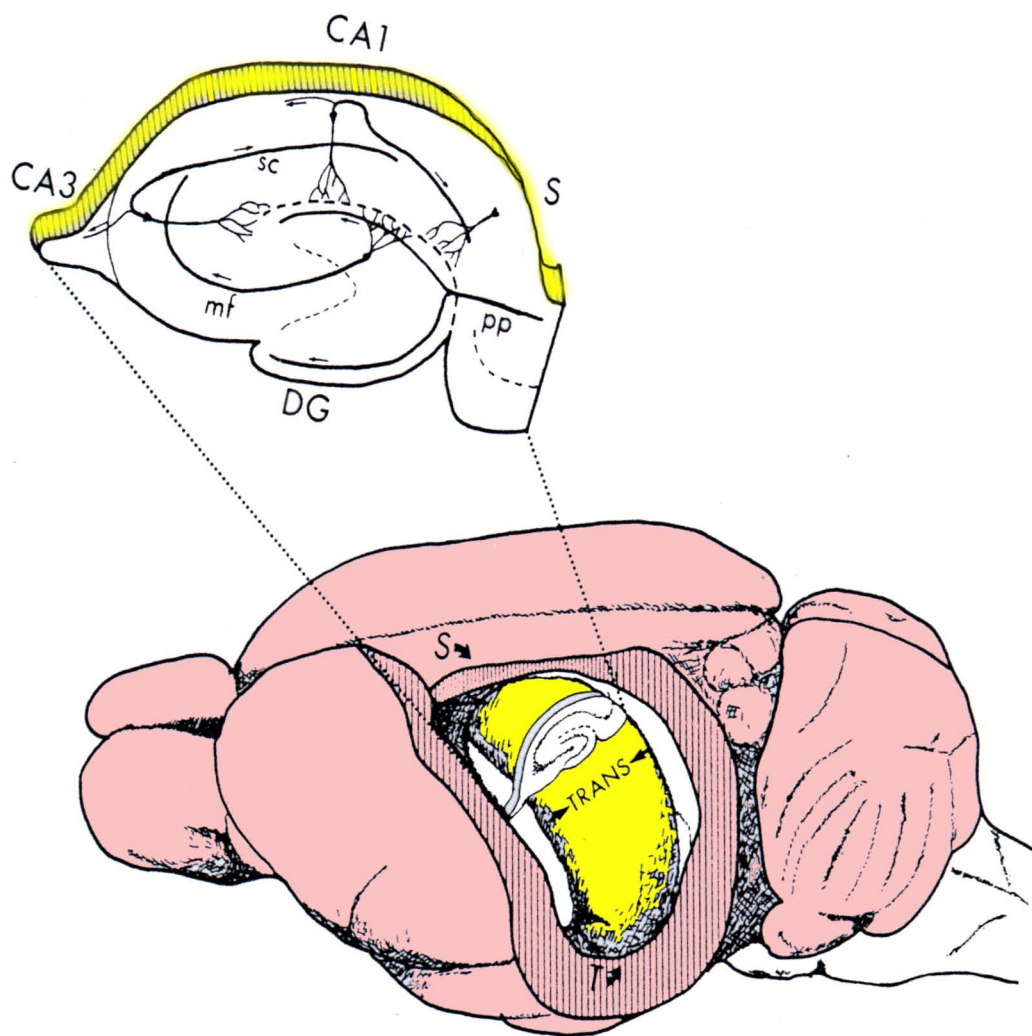
1996b; Chavis *et al.*, 1998; Shen and Slaughter, 1998; Stefani *et al.*, 1998c; Stefani *et al.*, 1998b), and potassium channels (Anwyl, 1999). In addition, they have been shown to modulate NMDA (Kelso *et al.*, 1992; Kinney and Slater, 1992; Behnisch and Reymann, 1993; Harvey *et al.*, 1993; Fitzjohn *et al.*, 1996; Mannaioni *et al.*, 1996; Pisani *et al.*, 1997b; Ugolini *et al.*, 1997; Cohen *et al.*, 1999; Ugolini *et al.*, 1999) and AMPA (O'Connor *et al.*, 1995; Ugolini *et al.*, 1997; Ugolini *et al.*, 1999) receptors as well as GABA release (Miles and Poncer, 1993; McBain, 1994; Jouvenceau *et al.*, 1995; Llano and Marty, 1995; Poncer and Miles, 1995; Poncer *et al.*, 1995; Schrader and Tasker, 1997; Jones *et al.*, 1998). A review of these processes is presented in Appendix B.

In 1997, Coyle reported in a minireview that the sum of NAAG research points towards a modulatory role for NAAG on excitatory neurotransmission via decreased glutamate release from presynaptic glutamatergic neurons caused by mGluR3 receptor activation and attenuation of postsynaptic NMDA receptor activation. I had at my disposal a model system that would allow me to address this hypothesis directly.

Research Rationale-*NAAG and LTP*

Most of the diseases mentioned previously are known to cause some form of memory impairment or loss. If one considers that the hippocampus is involved in certain forms of memory, one may speculate that NAAG may somehow be involved with the acquisition and/or the maintenance of certain types of memory. Many anatomical, physiological and behavioral studies have created a theoretical link between certain types of memory and the hippocampal formation (Johnston, 1998).

Figure 3. The hippocampal slice is a transverse section of hippocampal formation. This schematic shows the hippocampal formation in reference to the rat brain. In addition, the blow up shows a schematic of the acute hippocampal slice. In the acute slice, perforant path (pp) afferents from the entorhinal cortex synapse upon dendrites of the granule cells in the dentate gyrus. The axons of these granule cells, called mossy fibers (mf), project to the dendrites of CA3 pyramidal cells. The axons of these CA3 pyramidal cells, called Schaffer collaterals (sc), project to the dendrites of CA1 pyramidal cells. These paths make up what is generally referred to as the trisynaptic circuit of the hippocampal formation. It is important to note that the trisynaptic circuitry of the hippocampal formation is maintained in the acute hippocampal slice.

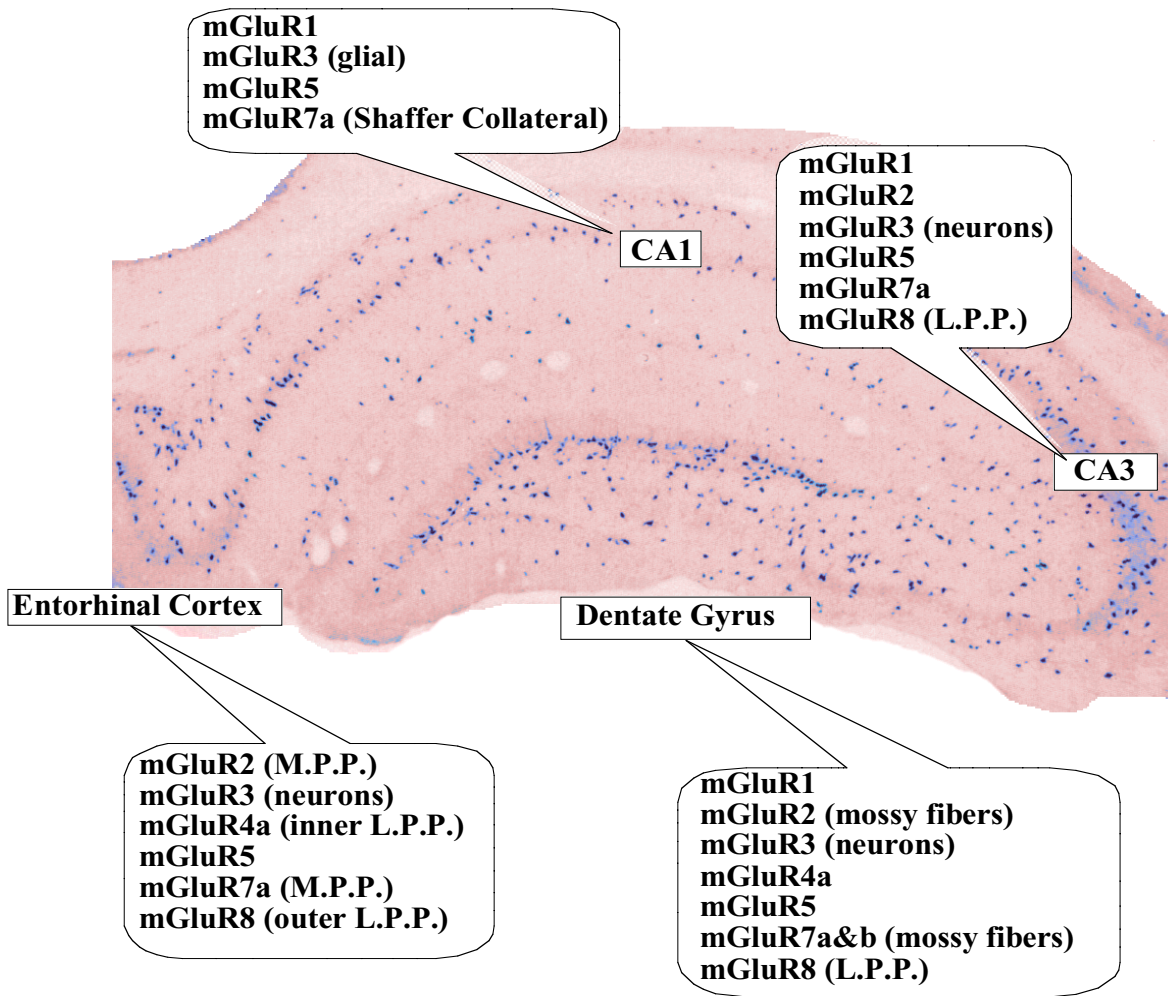CA1

CA3

S

sc

mf

pp

DG

S

TRANS

The hippocampal formation is a temporal lobe structure of the brain which consists of two interlocking C-shaped strips of cortex (the hippocampus and the dentate gyrus) in addition to the neighboring subiculum. The primary afferents leading into the hippocampal formation are from the entorhinal cortex through the lateral and medial perforant paths (LPP and MPP, respectively). These afferents synapse onto dendrites extending from the granule cells of the dentate gyrus creating what are called perforant path-granule cell (gc) synapses (MPP-gc or LPP-gc synapses). Transverse sections cut along the longitudinal axis of the hippocampal formation produce hippocampal slices (Figure 3). The hippocampal slice, maintained in an artificial cerebral spinal fluid (ACSF), remains richly endowed with neuronal activity for many hours and is ideal to study the role of NAAG. The various group I (mGluRs 1 and 5), II (mGluRs 2 and 3) and III (mGluRs 4a, 7a, 7b, and 8) subtypes of mGluRs, as well as NAAG and various subtypes of NMDA receptors, are expressed throughout the hippocampus (*mGluRs:* (Testa *et al*., 1994; Petralia *et al*., 1996b); *NAAG:* (Moffett and Namboodiri, 1995); *NMDA:* (Benke *et al*., 1995; Wenzel *et al*., 1995; Ozawa *et al*., 1998)).

In light of NAAG s anatomical distribution in the interneurons along the suprapyramidal blade of the dentate gyrus (Moffett and Namboodiri, 1995), and its specificity for the mGluR3 receptor (Wroblewska *et al.*, 1997), it is particularly interesting to find a heavy concentration of mGluR2/3 receptors on what is probably the glutamatergic afferents projecting from the entorhinal cortex into the midmolecular layer (medial perforant path; MPP; see (Testa *et al.*, 1994) for review; (Petralia *et al.*, 1996a;

Shigemoto *et al.*, 1997); Figure 4). In addition, mGluR2/3 anatomical distributions as

pictured in the review by Testa and colleagues (1994) show mGluR2/3 receptors located

Figure 4. Distribution of mGluRs and NAAG in the acute hippocampal slice. This figure

shows NAAG distribution throughout the acute hippocampal slice as well as the

distribution of various group I (mGluRs 1 and 5), II (mGluRs 2 and 3) and III (mGluRs

4a, 7a, 7b, and 8) subtypes of mGluRs. The hippocampal slice was modified from

Moffett and Namboodirii,(1995). The distribution of mGluRs was derived from (Petralia

et al., 1996b) and (Testa *et al*., 1994).

mGluR1
mGluR3 (glial)
mGluR5
mGluR7a (Shaffer Collateral)

CA1

mGluR1
mGluR2
mGluR3 (neurons)
mGluR5
mGluR7a
mGluR8 (L.P.P.)

CA3

Entorhinal Cortex

Dentate Gyrus

mGluR2 (M.P.P.)
mGluR3 (neurons)
mGluR4a (inner L.P.P.)
mGluR5
mGluR7a (M.P.P.)
mGluR8 (outer L.P.P.)

mGluR1
mGluR2 (mossy fibers)
mGluR3 (neurons)
mGluR4a
mGluR5
mGluR7a&b (mossy fibers)
mGluR8 (L.P.P.)

along the suprapyramidal blade of the dentate gyrus. Inhibitory interneurons and collaterals, modulate responses at the dendritic tree and cell body of the granule cells, as well as the presynaptic afferents at the MPP-gc synapse (Freund and Buzsaki, 1996).

Macek and colleagues (Macek *et al.*, 1996) showed that group II mGluRs are partially responsible for decreasing EPSPs in the midmolecular layer (MPP). In addition, they showed that group III mGluRs are partially responsible for decreasing EPSPs in the outer molecular layer (lateral perforant pathway; LPP). Unfortunately, they were unable to identify which of the two group II mGluR subtypes (mGluR2 and/or mGluR3) was responsible for decreasing the EPSPs in the MPP due to the lack of any specific antagonists to either mGluR2 or mGluR3. If we assume that NAAG stimulates the group II mGluR3 subtype on the midmolecular afferents (effectively decreasing glutamate release) then the similar anatomical distributions of NAAG and mGluR2/3 receptors in the hippocampus point toward a possible modulatory role in synaptic plasticity. One particular model used to study synaptic plasticity in the CNS is long-term potentiation.

Long-Term Potentiation

Alterations in neuronal activity after repetitive stimulation occur in what many consider a cellular model for learning and memory, long-term potentiation (LTP). When cells surrounding an extracellular recording electrode are depolarized from their resting state, but remain below threshold for an all-or-none action potential, the electrical activity recorded is a compilation of excitatory post-synaptic potentials (EPSPs). EPSPs are represented by a characteristic change in voltage measured around the tip of the

extracellular recording electrode. This post-synaptic electrical activity can be elicited both electrically and/or pharmacologically.

Two components making up the EPSPs of neuronal cells of the CNS, are the (S)-a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor mediated components. AMPA receptors, responsible for fast glutamatergic neurotransmission allow inward currents of sodium and outward currents of potassium. Once depolarization of the membrane releases Mg^{2+} from blocking the N-methyl-D-aspartate (NMDA) receptors, large inward calcium and sodium currents and outward potassium currents occur.

In 1973, Timothy Bliss and Terje Lomo (1973) showed that if a high-frequency stimulation (HFS), a train of electrical impulses, is given *via* a stimulating electrode placed into the perforant fiber pathway leading from the entorhinal cortex into the dentate gyrus, the EPSPs produced by a single subthreshold stimulation increase in both the granule cell body layer and the medial perforant pathway (for review see (Bliss and Lomo, 1973; Sarvey *et al.*, 1989). These EPSPs can remain increased or potentiated for hours in the hippocampal slice (Alger and Teyler, 1976; Andersen, 1977; Stanton and Sarvey, 1984; Stanton and Sarvey, 1985c). This prolonged potentiation of excitatory postsynaptic potentials is called long-term potentiation (LTP).

Since Bliss and Lomo published their landmark paper, LTP has been characterized in multiple systems. The underlying mechanisms behind the establishment of LTP are presumably synaptic. To date, the controversy remains as to whether LTP is a pre- and/or post-synaptic strengthening phenomenon (Malenka and Nicoll, 1999).

The Hebbian Synapse

The electrophysiologist, and the neural network engineer, asked to discuss the conditions that occur during the strengthening of a pre- to postsynaptic continuity, usually refer to a simple, but elegant, postulate put forward in the late 1940s by Donald Hebb, a Canadian psychologist.   That is:

 When an axon of cell A is near enough to excite cell B repeatedly or

 consistently takes place in firing it, some growth process or metabolic

 change takes place in one or both cells such that A s efficiency, as one of

 the cells firing B, is increased. - (Hebb, 1949)

The Hebbian synapse , which requires depolarization of the postsynaptic neuron during simultaneous stimulation of the presynaptic neuron, have been discovered for example, in the neocortex and hippocampus, and are the types of synapses seen in the model of LTP discussed in this dissertation.

LTP of the EPSPs recorded at the MPP has been shown to require the activation of NMDA receptors (Harris and Cotman, 1986; Herron *et al.*, 1986; Burgard *et al.*, 1989), an increase in cAMP levels (Stanton and Sarvey, 1985a; Blitzer *et al.*, 1995; Nguyen and Kandel, 1996; Blitzer *et al.*, 1998), β-adrenergic receptor activation (Stanton and Sarvey, 1985b; Bramham *et al.*, 1997), protein kinase A (Castro-Alamancos and Calcagnotto, 1999; Linden and Ahn, 1999) and C activation (Malinow *et al.*, 1989), receptor tyrosine

kinase activation (O'Dell *et al.*, 1991; Voulalas, 1997), and the Ras-MAPK signaling

pathway (Voulalas, 1997; Orban *et al.*, 1999).

Both the NMDA (Herron *et al*., 1986) and group II mGluR receptors have been

implicated in synaptic plasticity (Huang *et al*., 1997a; Huang *et al*., 1997b; Huang *et al*.,

1999a; Huang *et al*., 1999b). Since NAAG effectively competes with glutamate for the

post-synaptic NMDA receptor, and presynaptic group II mGluR activation decreases

neurotransmitter release from the presynaptic cell, I predict that *NAAG will prevent high-*

frequency-induced increases in EPSPs recorded in the granule cell body layer and the

medial perforant path-granule cell dendritic layer. By measuring MPP-gc EPSPs in the

hippocampal slice granule cell body layer of the dentate gyrus, I was able to assess the

influence of NAAG upon LTP by comparing recordings before and after drug perfusion.

Research Rationale-*NAAG and epileptiform activity*

Epilepsy is a disease that strikes about 1 in 100 people in the United States. It is a

brain disorder in which clusters of neurons no longer signal in a normal pattern. For

example, during a seizure event, the neurons which normally fire at a rate of about 80

times per second may fire up to 500 times per second [NIH Publication N0. 00-156;

March 2000]. A better understanding of what occurs at the pre- and postsynaptic neurons

during epileptiform bursting is essential.

The group II mGluR receptors have been shown to play a role in epilepsy (Merlin

et al., 1995). Interestingly, Najm and colleagues show that in the rat model of temporal

lobe epilepsy, kainic-acid (KA) induces changes in the relative ratios of N-acetyl groups

(NAAG and N-acetyl-aspartate (NAA)) to creatine (Najm *et al.*, 1997). During the ictal

phase, the ratio increases. During the postictal and interictal phases, the ratio decreases.

In addition, a summary of the findings of Meyerhoff and colleagues. (Meyerhoff *et al.*,

1985; Meyerhoff *et al.*, 1989; Meyerhoff *et al.*, 1992a; Meyerhoff *et al.*, 1992b;

Meyerhoff *et al.*, 1992c), include kindling-induced increases in NAAG in entorhinal

cortex and seizure-induced decreases in GCPII activity. These results suggest that

NAAG may be involved in the etiology of epilepsy.

Hippocampal dentate gyrus granule cells provide a 'gate' for neuronal activity to

spread from glutamatergic and non-glutamatergic afferents into the hippocampus. Thus,

the dentate gyrus can act as a control point for epileptigenesis in the hippocampal

formation as well as other downstream sites to which the hippocampus sends its efferent

projections (Lothman *et al.*, 1992). Because NAAG levels are shown to increase in KA

and kindling-induced models of epilepsy, and NAAG, the NMDA and the mGluR3

receptors are distributed in the dentate gyrus, I hypothesized that NAAG could influence

the bursting activity seen in granule cells.

During my study of the role of NAAG on LTP, I discovered that the β-isomer of

NAAG (β-NAAG) is a specific antagonist to the mGluR3 receptor. In addition, I showed

that NAAG did not affect the NMDA receptor. These findings provided me with an

opportunity to investigate a possible role for NAAG and the mGluR3 receptor in

epileptiform bursting in the granule cells of the dentate gyrus. Coupled to my findings

that NAAG blocked LTP at the MPP-gc synapse I predicted that *exogenously applied*

NAAG can reduce epileptiform activity recorded in the dentate gyrus. By measuring

MPP-gc EPSPs and population spikes in the hippocampal slice granule cell body layer of

the dentate gyrus, I was able to assess the influence of NAAG upon epileptiform activity

by comparing recordings before and after drug perfusion.

Specific Aims

The specific aims of this study were to:

1) conclude my preliminary investigation of NAAG-evoked neuromodulation of long-

   term potentiation (LTP) at the medial perforant path-granule cell (MPP-gc) synapse

   in the dentate gyrus of the rat hippocampus;

2) investigate a possible antiepileptic role of NAAG against induced epileptiform

   activity in the hippocampal slice granule cell body layer.

This dissertation is comprised of two manuscripts, presented as two sections, and a

general discussion at the end. All references will be placed at the end of this dissertation.

All work presented in this dissertation is original. All experiments were conducted by

myself except the transfection studies in section one which were done in collaboration

with Joe Neale, Ph.D. and Barbara Wroblewska, Ph.D. and the blind whole-cell patch

recordings which were done with John Sarvey, Ph.D.

β-NAAG Rescues LTP from Blockade by NAAG in Rat Dentate Gyrus via the Metabotropic Glutamate

Receptor Subtype mGluR3

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Abstract

N-Acetylaspartylglutamate (NAAG) is an agonist at the type 3 metabotropic glutamate

receptor (mGluR3), which is coupled to a $G_{i/o}$ protein. When activated, the mGluR3

receptor inhibits adenylyl cyclase and blocks the cAMP second messenger cascade.

Long-term potentiation (LTP) in the medial perforant path (MPP) of the hippocampal

dentate gyrus requires increases in cAMP. The presence of mGluR3 receptors and

NAAG in neurons of the dentate gyrus leads to the hypothesis that this peptide

transmitter may inhibit LTP in the dentate gyrus. High frequency stimulation (100 Hz;

2s) of the MPP resulted in LTP of extracellularly recorded excitatory postsynaptic

potentials at the MPP-granule cell synapse of rat hippocampal slices. Perfusion of the

slice with NAAG (50 and 200 M) blocked LTP. Paired-pulse depression of the EPSP

at 20 and 80 ms interpulse intervals (IPI) indicates that the effects of NAAG are not

presynaptic; neither concentration affected N-methyl-D-aspartate receptor currents in the

granule cells of the acute hippocampal slice. The group II mGluR antagonist ethyl

glutamate (100 M) and a structural analogue of NAAG, β-NAAG (100 M), prevented

the blockade of LTP by NAAG. Paired-pulse depression at 20 and 80 ms IPI indicates

that the effects of β-NAAG are not presynaptic. β-NAAG did not affect inositol

triphosphate production stimulated by the agonist glutamate in cells expressing the group

I mGluR1α or mGluR5. β-NAAG blocked the decrease in forskolin-stimulated cAMP

by the group II mGluR agonist DCG-IV but not the group III mGluR agonist L-AP4, in

cerebellar granule cells. In cells transfected with mGluR3, but not mGluR2, β-NAAG

blocked forskolin-stimulated cAMP responses to glutamate, NAAG, the nonspecific

group I, II agonist *trans*-ACPD, and the group II agonist DCG-IV. We conclude that β-

NAAG is a selective mGluR antagonist capable of differentiating between mGluR2 and

mGluR3 subtypes, and that the mGluR3 receptor functions to regulate activity-dependent synaptic potentiation in the hippocampus.

Introduction

Since its discovery in 1964 (Curatolo, 1964), the vertebrate neuropeptide, N-acetyl-L-aspartyl-L-glutamate (NAAG) has been shown to be widely distributed in neurons throughout the mammalian nervous system (Anderson *et al.*, 1986; Forloni *et al.*, 1987; Tieman *et al.*, 1987; Williamson and Neale, 1988b; Tieman *et al.*, 1991; Moffett *et al.*, 1993; Moffett *et al.*, 1994; Moffett and Namboodiri, 1995; Tieman and Tieman, 1996; Renno *et al.*, 1997). NAAG meets each of the traditional criteria for a neurotransmitter (for review see (Coyle, 1997; Neale, 2000). NAAG is a selective agonist at the type 3 metabotropic glutamate receptor (mGluR3) in neurons, glia and transfected cells, where it has a potency similar to that of glutamate (Wroblewska *et al.*, 1993; Wroblewska *et al.*, 1997; Wroblewska *et al.*, 1998). The peptide is also a low potency agonist at the NMDA receptor (Westbrook *et al.*, 1986; Trombley and Westbrook, 1990; Valivullah *et al.*, 1994). Additional data suggest that NAAG may act as a partial agonist at this receptor (Puttfarcken *et al.*, 1993; Grunze *et al.*, 1996), although this has yet to be rigorously tested.

The inactivation of synaptically released NAAG is achieved by a membrane-bound (Riveros and Orrego, 1984; Robinson *et al.*, 1987) peptidase on the extracellular face of glia (Cassidy and Neale, 1993). This glutamate carboxypeptidase II (GCP II) was found to be identical to prostate specific membrane antigen in humans (Carter *et al.*, 1996) and has been cloned from rat nervous system libraries (Bzdega *et al.*, 1997; Luthi-Carter *et al.*, 1998). GCP II activity is inhibited by quisqualate ($K_i = 2$ M), phosphate (IC50 = 100 M), sulfate (IC50 =1 mM) and β-NAAG ($K_i = 1$ M) , a structural analogue of NAAG in which the peptide bond is formed by the β-carboxyl group of

aspartate (Robinson *et al.*, 1987; Serval *et al.*, 1990). The distribution of both NAAG and GCP II are altered in human degenerative diseases (see (Coyle, 1997) for review), although it is not possible from these data to resolve primary from secondary degenerative tissue changes.

Acting at the mGluR3 receptor, NAAG causes a decrease in forskolin-stimulated cAMP levels in neurons, glia and cultured mammalian cells stably expressing mGluR3 cDNAs (Wroblewska *et al.*, 1993; Wroblewska *et al.*, 1997; Wroblewska *et al.*, 1998). The mGluR3 is a member of the group II metabotropic glutamate receptors, which have been implicated in suppression of voltage-dependent calcium conductance in cerebellar granule cells (Chavis *et al.*, 1994), neocortical neurons (Sayer *et al.*, 1992) and amphibian olfactory neurons (Bischofberger and Schild, 1996). The group II agonist, DCG-IV, which also inhibits voltage-dependent calcium currents, has been reported to suppress synaptic transmission to motor neurons in the spinal cord (Ishida *et al.*, 1993), potassium-induced release of GABA in cortical cell cultures (Schaffhauser *et al.*, 1998), and transmission at the mossy fiber-CA3 synapse in the hippocampus (Kamiya *et al.*, 1996) and a reciprocal synapse in the accessory olfactory tract (Hayashi *et al.*, 1993). In cerebellar slices, exogenously applied NAAG suppressed the excitatory response of Purkinje cell dendrites to climbing fiber activation (Sekiguchi *et al.*, 1989). These data have led to the hypothesis that one function of NAAG following synaptic release is to activate presynaptic mGluR3s to inhibit subsequent transmitter release.

The rat hippocampal slice is a preparation richly endowed with ionotropic and metabotropic glutamate receptors, including mGluR3 (Shigemoto *et al.*, 1997). NAAG is concentrated in hippocampal interneurons (Anderson *et al.*, 1986; Moffett *et al.*, 1993;

Moffett and Namboodiri, 1995), and GCP II activity is found throughout the

hippocampus (Fuhrman *et al.*, 1994; Bzdega *et al.*, 1997; Luthi-Carter *et al.*, 1998).

Group II metabotropic glutamate receptors (mGluR2 and mGluR3) have been localized

to the suprapyramidal blade of the dentate gyrus in apparent association with the medial

perforant path from entorhinal cortex to the midmolecular layer (Testa *et al.*, 1994),

(Petralia *et al.*, 1996a). Inhibitory interneurons and collaterals, located in the dentate

gyrus, modulate responses at the dendritic tree and cell body of the granule cells, as well

as the presynaptic afferents at the medial perforant path-granule cell synapse (Freund and

Buzsaki, 1996).

Hippocampal LTP has been shown to require NMDA receptor activation (Harris

and Cotman, 1986; Herron *et al.*, 1986; Burgard *et al.*, 1989) and an increase in cAMP

levels (Stanton and Sarvey, 1985c; Blitzer *et al.*, 1995; Nguyen and Kandel, 1996; Blitzer

et al., 1998). In the dentate gyrus, LTP also requires β-adrenergic receptor activation

(Stanton and Sarvey, 1985b; Bramham *et al.*, 1997). Because NAAG decreases

forskolin-stimulated cAMP levels via the mGluR3 receptor (Wroblewska *et al.*, 1993;

Wroblewska *et al.*, 1997; Wroblewska *et al.*, 1998) and NAAG and mGluR3 are present

in the dentate gyrus, we speculate that NAAG may have a modulatory role on synaptic

plasticity in the medial perforant path-granule cell (MPP-gc) synapse. Consistent with a

role for NAAG and mGluR3 in hippocampal plasticity, activation of group II mGluRs

has been reported to decrease excitatory postsynaptic potentials (EPSPs) in the mid-

molecular layer (Macek *et al.*, 1996; Kilbride *et al.*, 1998), and the group II mGluR

agonist, DCG- IV, blocks induction of LTP in the dentate gyrus (Huang *et al.*, 1997b).

Additionally, DCG-IV and NAAG have been reported to induce long-lasting depression

in the medial perforant path of the disinhibited dentate gyrus (Huang *et al.*, 1999b), and

NAAG reduces LTP of inhibitory post-synaptic potentials in the recurrent inhibitory

circuit following alvear stimulation in CA1 (Grunze *et al.*, 1996). In the course of testing

the hypothesis that NAAG affects synaptic plasticity in the hippocampus, we found that

β-NAAG functions as an mGluR3 antagonist. We are the first to report this and to report

a specific role for the mGluR3 receptor in LTP.

Materials and Methods

Both α- and β-isomers of N-acetylaspartylglutamate, and adenosine triphosphate were purchased from Sigma. *trans*-ACPD (trans-1-aminocyclopentane-1,3-dicarboxylate), DCG-IV ((2S,2 R,3 R)-2-(2 ,3 -dicarboxycyclopropyl)glycine), ethyl glutamate, AP4 (L(+)-2-amino-4-phosphonobutyric acid), MK801, CNQX and CGP-55845 were purchased from Tocris Cookson Inc. Tissue culture reagents were obtained from Gibco and Biofluids. Leupeptin was obtained from Boehringer Mannheim, and QX-314 from Astra Pharmaceuticals.

Preparation of hippocampal slices-Male Sprague-Dawley rats (Taconic, Germantown, N.Y.) weighing 80-210 g were anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and decapitated. Experiments were conducted according to the principles set forth in the "Guide for Care and Use of Laboratory Animals," Institute of Animal Resources, National Research Council, NIH Pub. No. 74-23. Transverse slices (400 m) of hippocampus were prepared using a McIlwain tissue chopper. Slices were placed in a modified Oslo interface recording chamber at 32-34¡ C and perfused at a rate of 3 ml/min with artificial cerebrospinal fluid (ACSF) containing 26 mM $NaHCO_3$, 124 mM NaCl, 1.75 mM KCl, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4mM $CaCl_2$, and 10 mM dextrose; pH was adjusted to 7.4 by bubbling with a 95% CO_2/5 % O_2 gas mixture. Slices were allowed to equilibrate for at least two hours before recordings were initiated.

Electrophysiology- Figure 1A is a schematic of the hippocampal slice showing the placement of the stimulating electrode in the medial perforant path (MPP) leading from

the entorhinal cortex to the dentate gyrus. Recording electrodes were placed in the MPP and in the granule cell layer located along the suprapyramidal blade of the dentate gyrus. In subsequent figures, the initial negative slope of the EPSP, recorded in the MPP, is plotted as percent of baseline EPSP slope (mean – SEM).

Stimuli were delivered to the MPP fibers through a 100 m diameter, monopolar, Teflon-insulated, stainless steel wire electrode, exposed only at the tip. Extracellular recordings were obtained using glass micropipettes filled with 2M NaCl, 2-6 MΩ resistance. Recording electrodes were positioned a minimum of 500 m from the stimulating electrodes, and lowered to a final depth of 80 m into the slice. Only slices showing complete abolition of the population spike, recorded in the cell body layer, at 20 ms interpulse intervals in a paired-pulse paradigm, were selected for study. Isolation of medial perforant path responses was confirmed by paired-pulse depression of the EPSP seen at an 80 ms interpulse interval using a current intensity that elicited an EPSP that was just subthreshold for a reflected spike (McNaughton, 1980; Bramham *et al.*, 1997). Test stimuli were delivered to the mid-molecular layer of the dentate gyrus every 30s to evoke subthreshold EPSPs. After establishment of a stable baseline recording, EPSPs and population spikes were recorded extracellularly from the medial perforant path and the granule cell body layer in the dentate gyrus. Application of pharmacological agents was achieved by switching the chamber perfusion solution to ACSF containing the drug.

Drugs were tested for possible effects on presynaptic release of glutamate using a paired-pulse paradigm. Two subthreshold stimuli (10 sec) were given to the MPP at interpulse intervals of 20, 30 and 80 msec. The initial slope of the second EPSP was normalized to the slope of the first EPSP of a pair (as shown in (Bramham *et al.*, 1997).

Blind whole-cell patch clamp recording was used to measure AMPA and NMDA receptor mediated current in granule cells of the acute hippocampal slice (Blanton *et al.*, 1989). Patch pipettes (4-8 MΩ) were pulled in two stages (Model p-80/PC Flaming-Brown Micropipette Puller; Sutter Instruments Company) and filled with a solution of: 140 mM CsF, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM TEA, 5mM Na_2-phosphocreatine, 5 mM QX-314**,** 0.1 mM Leupeptin and 4 mM Na_2-ATP; pH was adjusted to 7.35 with CsOH. Osmolality was adjusted to 290 mOsmole/1000g with sucrose. During patch experiments, 20 μM bicuculline and 50 nM CGP-55845 were added to the ACSF. The whole-cell configuration was established in voltage-clamp mode approximately 10 minutes after establishing a seal in the cell-attached configuration (Hamill *et al.*, 1981). Current measurements were made at holding potentials of -80, -30 and +20 mV (Axopatch 1D, Digidata 1200 series interface, pClamp 8.0). The late EPSC component (NMDA receptor current) was separated from the early EPSC component (AMPA receptor current; (Hestrin *et al.*, 1990) by taking current measurements approximately 40 ms after stimulus. Resting membrane potential was also measured at the time of whole-cell formation. Cells with resting potentials near —80 mV were accepted for data analysis. All other conditions of our preparations were the same as those described during extracellular recording.

Cell Cultures-Primary cultures of rat cerebellar granule cells were prepared from 8-day old Sprague-Dawley pups (Taconic, Germantown, N.Y.) as described previously (Gallo *et al.*, 1982). Cells were plated on the poly-L-lysine coated dishes, at the density 1.25 x 10^6cells/ml and cultured in basal Eagle s medium supplemented with 10 % fetal bovine

serum (heat inactivated), 2 mM glutamine, 50 mg/ml gentamicin, and 25 mM KCl. To prevent proliferation of the non-neuronal cells cytosine arabinoside was added (10 M) to the culture 24 hours after plating. Cells kept in vitro for 6-8 days were used.

Transfected cell lines-Mammalian cell lines expressing metabotropic glutamate receptor mRNAs were prepared as described previously (Wroblewska *et al.*, 1997). Briefly, mGluR2 and mGluR3 cDNAs (kindly provided by Dr. S. Nakanishi) were inserted into an EcoRI site of the mammalian expression vector pcDNA3 (Invitrogen) containing a neomycin-resistant gene. Mammalian cells (Chinese Hamster Ovary cells, CHO, and Baby Hamster Kidney cells, BHK) were transfected with these constructs using the calcium phosphate method, as described by Chen and Okayama (Chen and Okayama, 1987). The BHK cell lines were maintained in the DMEM medium (Gibco) with L-glutamine (2 mM), sodium pyruvate, 10 % fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (pen/strep, Biofluids). The CHO cells were maintained in the medium described previously (Tanabe *et al.*, 1992). Gentamycin (G-418, Gibco) was used to select stable neomycin-resistant cell lines expressing mGluR mRNAs. Positive clones were identified by reverse transcription PCR reaction with specific primers (as described in (Wroblewska *et al.*, 1997) and the response to forskolin stimulated cAMP formation, as described previously (Wroblewska *et al.*, 1997).

Assays-IP3: Cells were prepared and assayed for IP3 using methods previously described (Wroblewska *et al.*, 1993). *cAMP:* Rat cerebellar granule cells, BHK-mGluR3 or CHO-mGluR2 stably transfected cell lines, were seeded on the 24-well plates

and grown for 6-8 days in the growing medium. Cells were preincubated for 10 min in medium containing 1 M MK801, 10 M CNQX, and either 500 M IBMX for granule cells, or PBS and IBMX for cell lines. Cells were then incubated for 7 min with either forskolin (10 M) alone, forskolin + agonist, or forskolin + agonist + β-NAAG (100 M), as described previously (Wroblewska *et al.*, 1993; Wroblewska *et al.*, 1997). The measurements of cAMP were performed with Amerlex cAMP ^{125}I kit (Amersham). Curve fitting of the data was performed using GraphPad Prism 2.0 (GraphPad Software, Inc.)

Data analysis and statistics- 1) LTP-extracellular recordings: responses were amplified, filtered (d.c.-3 kHz), digitized at 20kHz (DAS-20 interface, Keithley Metrabyte, Taunton, MA) and stored for analysis using the Labman data acquisition analysis program (a gift of Dr. T. Teyler, NeuroScientific Laboratories, Rootstown, OH). Measures of synaptic efficacy were made using the initial slope of the EPSP. Values were normalized to percentages of the mean baseline value. Data are expressed as the mean–SEM. Statistical analyses of drug effects in hippocampal slices were carried out in Statview (Abacus Concepts, Inc., Berkeley, CA) using a two-tailed paired t-test or ANOVA plus a post hoc Bonferroni/Dunn test for multiple comparisons. *2) Whole cell patch clamp recordings:* responses were amplified, digitized at 20kHz and stored for analysis (Axopatch 1D, Digidata 1200 series interface, pClamp 8.0). Measurements of NMDA current were made when AMPA current subsided (approximately 40 ms). *3) Biochemistry:* Statistical analyses of drug effects in cultured cells were carried out in Sigma Plot using the Student's t-test. Data were fit to a sigmoidal log dose-response

curve using Prism 2.0 (GraphPad Software, Inc). A probability of 0.05 was selected as

the level of statistical significance for all data.

Figure 1. NAAG blocks LTP. *A)* Schematic representation of the rat hippocampal slice. Extracellular recording electrodes placed in the granule cell layer and in the mid-molecular layer (medial perforant path; MPP) of the dentate gyrus record characteristic potentials during MPP stimulation. In this figure, we show a subthreshold EPSP recorded in both the MPP and granule cell layer. Higher stimulation intensity elicited a population spike (not shown). *B)* LTP induced by high frequency stimulation (HFS; 100 Hz, 2 sec) at 0 minutes lasted more than 2 hours (ACSF; N=5; p<0.05; paired t-test). Perfusion of 200 M NAAG 20 minutes prior to HFS blocked LTP (NAAG; N=4; p>0.05; paired t-test). In B, the upper inset (ACSF) shows a representative baseline EPSP prior to HFS, overlaid with the EPSP after the HFS. The lower inset (NAAG) shows a representative baseline EPSP prior to HFS, overlaid with an EPSP 60 minutes after HFS. *C)* Perfusion of 50 M NAAG 20 minutes prior to HFS blocked maintenance of LTP (NAAG; N=3; p>0.05; paired t-test). The initial negative slope of the EPSP recorded in the MPP is plotted as percent of baseline EPSP slope. Each data point represents the mean – SEM. The horizontal bar above the abscissa is the bath perfusion time for NAAG.
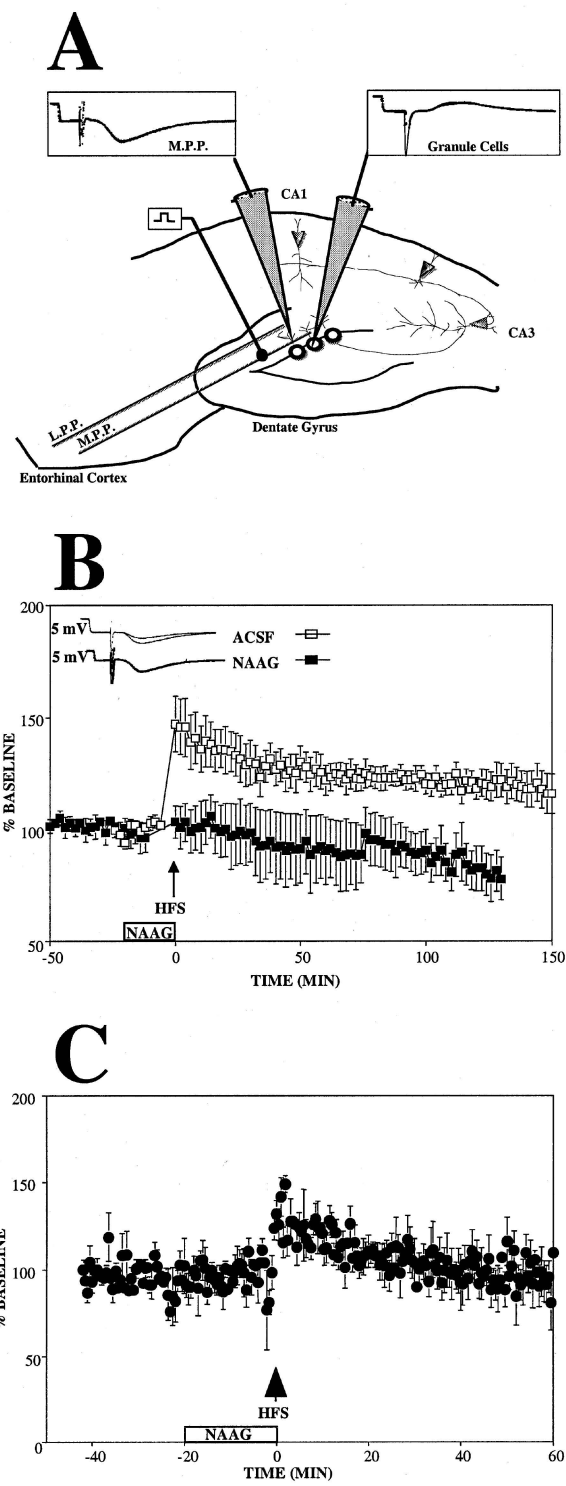
Figure 1

Results

NAAG blocks LTP

High frequency stimulation (HFS; 100 Hz, 2 sec) of the medial perforant path (MPP) increased subthreshold EPSP slopes recorded in the MPP (Figure 1B; see upper tracing in inset). Long-term potentiation (LTP; approximately 120% baseline) was significant 120 minutes after HFS (Figure 1B; N=5; p<0.05; paired t-test). To test the prediction that NAAG will block LTP in the dentate gyrus, we bath-perfused 200 M NAAG for 20 minutes prior to giving HFS (100 Hz, 2 sec). NAAG perfusion had no detectable effect on EPSP slope or amplitude during the acquisition of baseline (see lower tracing in inset Figure 1B; N=4; p>0.05; ANOVA). 200 M NAAG blocked the increase in slope and amplitude normally seen in control LTP (N=4; p>0.05; paired t-test; Figure 1B). To test the effects of a lower concentration of NAAG on LTP, we bath perfused 50 M NAAG for 20 minutes prior to giving HFS (100 Hz, 2 sec). As predicted, 50 M NAAG prevented the maintenance phase of LTP (N=3; p>0.05; paired t-test; Figure 1C). The ability to obtain post-tetanic potentiation at a lower concentration of NAAG, but not with 200 M NAAG suggested that the higher concentration of NAAG was in some way affecting presynaptic release of transmitter. This was supported by the findings of Macek and colleagues (Macek *et al.*, 1996) who report that group II mGluR autoreceptors decrease EPSPs at the MPP-gc synapse. In contrast, Huang and colleagues (Huang *et al.*, 1997b), reported that NAAG acts at a postsynaptic site. In light of these previous findings, further analysis of the effects of NAAG needed to be performed.

Figure 2. NAAG has no effect on NMDA receptor mediated current in dentate gyrus granule cells. *A)* Neither 50 nor 200 μM NAAG affected NMDA receptor activation in dentate gyrus granule cells of intact 400μm thick hippocampal slices. EPSCs were elicited at potentials of —80, -30 mV or +20 mV (N=3). Measurements were taken approximately 40 ms after stimulation (see vertical bar in inset) to isolate NMDA receptor current from AMPA receptor current. The inset shows representative currents at -80, -30 and +20 mV (250 ms voltage steps from —80 mV holding potential; abscissa). Data are from EPSCs in the absence of, and after 20 minutes exposure to NAAG.
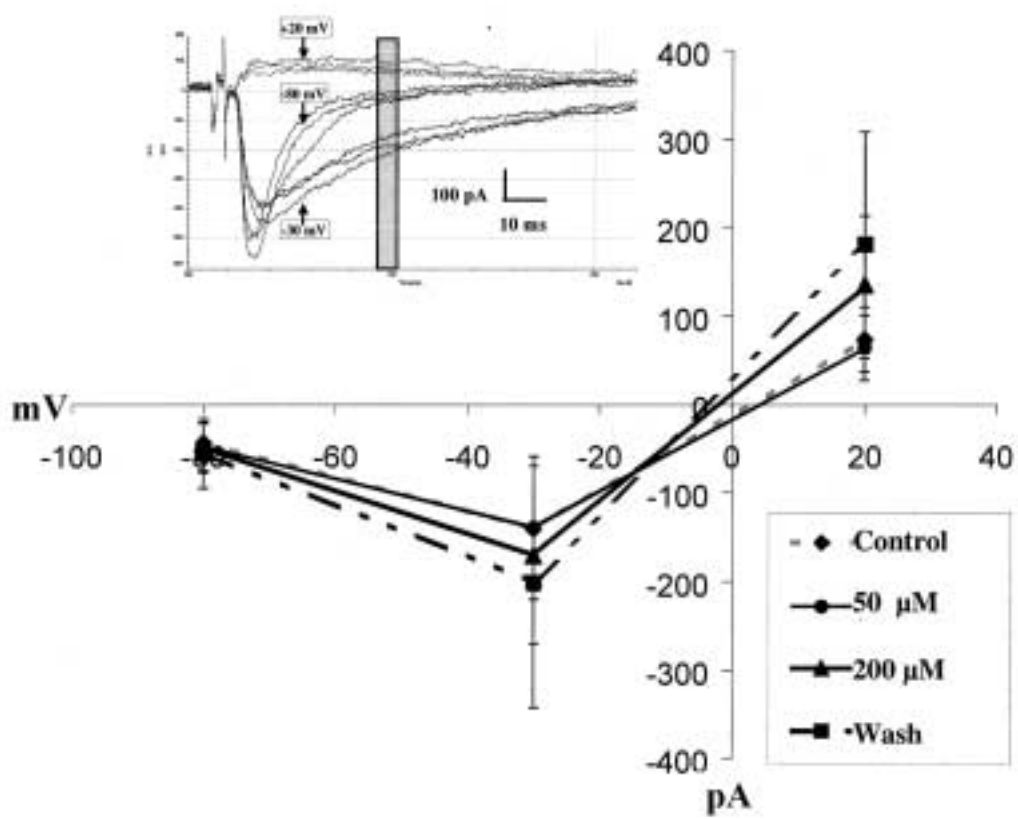
Figure 2

NAAG has no effect on NMDA or AMPA receptor current

To confirm that NAAG did not affect NMDA or AMPA receptor currents in granule cells in the acute hippocampal slice, we used blind whole cell patch clamp recordings of EPSCs. Neither 50 nor 200 M NAAG had any effect on the NMDA receptor-mediated slow EPSC, or on the AMPA receptor-mediated fast EPSC at any potential (N=3; Figure 2). We conclude from these experiments that the ability of higher concentrations of NAAG to inhibit the post-tetanic potentiation seen with 50 M NAAG is not caused by NAAG acting upon either NMDA or AMPA receptors.

Ethyl glutamate and β-NAAG rescue LTP from NAAG

To further characterize the ability of NAAG to block LTP, we tested the ability of the group II mGluR specific antagonist ethyl glutamate to antagonize the effects of NAAG. Ethyl glutamate prevented the blockade of LTP by NAAG (Figure 3A; N=3; p<0.05; paired t-test). This supports our hypothesis that NAAG is acting through either the group II mGluR2 or mGluR3 subtype.

Because NAAG can be hydrolyzed by glutamate carboxypeptidase II (GCP II), we used β-NAAG (β−isomer of NAAG) to inhibit the enzyme prior to perfusion of NAAG. While β-NAAG is an inhibitor (K_i = 1 M) of the extracellular peptidase (glutamate carboxypeptidase II; GCP II), β-NAAG, similar to ethyl glutamate, prevented the blockade of LTP by NAAG (Figure 3B; N=4; p<0.05; paired t-test). Interestingly, the concentrations of PO_4^{2+} and SO_4^{2+} (1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$) in our ACSF were high enough to inhibit the GCP II (IC50=100 M and 1mM, respectively;

(Robinson *et al.*, 1987). These data suggest that β-NAAG rescues LTP *via* the group II mGluRs.

Figure 3. Ethyl glutamate and β-NAAG rescue LTP from NAAG. *A)* 100 M ethyl glutamate administered for 20 minutes prior to, during, and for 20 minutes after 50 M NAAG perfusion, rescued LTP in the medial perforant path of the dentate gyrus. The EPSP was significantly increased from baseline 120 minutes post-HFS (100 Hz, 2 sec; N=3; p<0.05; paired t-test). The upper and lower horizontal bars along the abscissa represent the infusion time for NAAG and ethyl glutamate, respectively. *B)* 100 M β-NAAG administered 20 minutes prior to, during, and after 200 M NAAG perfusion, rescued LTP in the medial perforant path of the dentate gyrus. LTP was significantly increased from baseline 120 minutes post-HFS (100 Hz, 2 sec; N=4; p<0.05; paired t-test). The upper and lower horizontal bars along the abscissa represent the infusion time for NAAG and β-NAAG, respectively. Inset tracings, taken just prior to and 60 and 120 minutes after the HFS, show an increase in EPSP slope after HFS. *C)* 100 M β-NAAG perfusion for 60 minutes, without giving HFS, had no significant effect on EPSP slope measured just prior to, 50 and 100 minutes after perfusion was begun (N=16; p>0.05; ANOVA). The horizontal bar above the abscissa indicates bath infusion of β-NAAG. *D)* Neither 50 M NAAG (N=3) nor 100 M β-NAAG (N=16) affected paired-pulse depression measured at 20 and 80 msec interpulse intervals (p>0.05; paired t-test). The slope of the second EPSP is plotted as percent of maximum slope of the first EPSP of each pair. Each data point represents the mean – SEM.
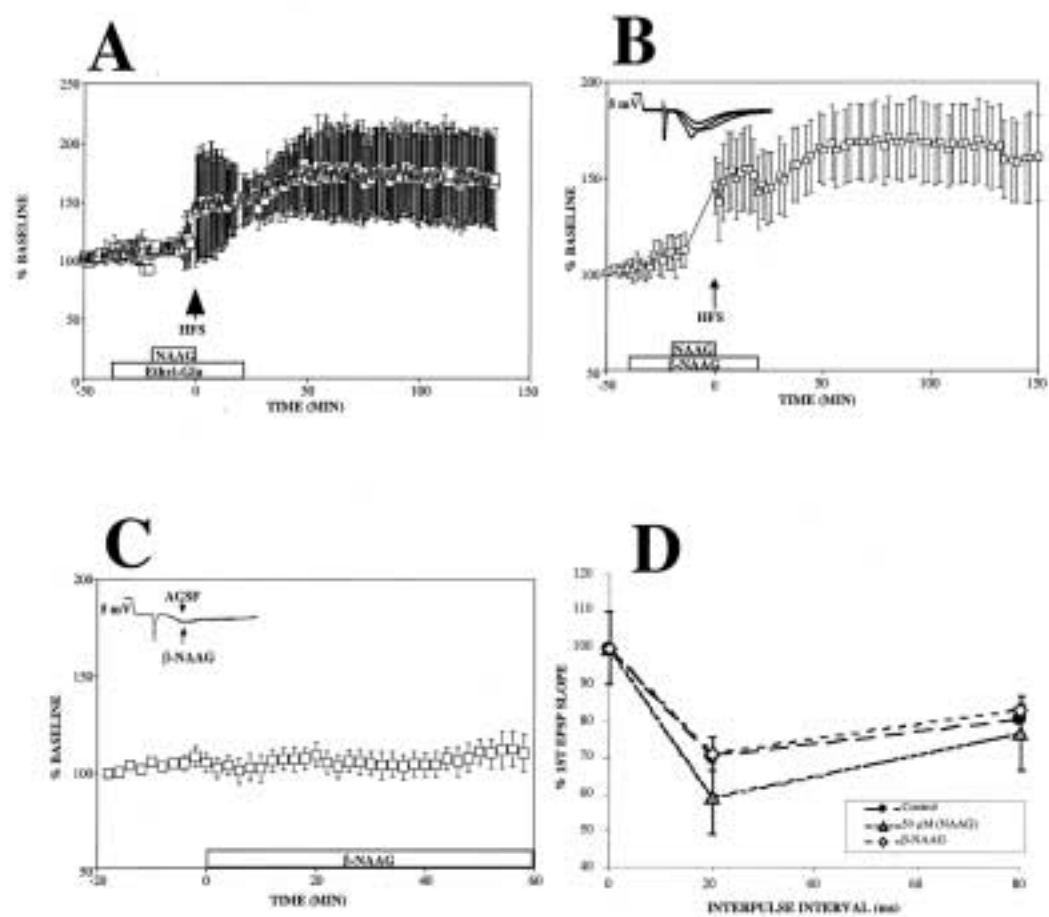
Figure 3

β-NAAG has no effect on EPSPs

To characterize the response of the hippocampal slice to β-NAAG, we bath-perfused 100 M β-NAAG for 60 minutes without administering HFS. Our results showed that β-NAAG had no effect on MPP EPSPs (Figure 3C; N=16; p>0.05; ANOVA).

β-NAAG and NAAG have no effect on presynaptic transmitter release

Due to β-NAAG s ability to block the effects of NAAG, and in light of the findings of Huang and colleagues (1999), we predicted that β-NAAG also acts at a postsynaptic site. We utilized a typical paired-pulse paradigm (McNaughton, 1982) to confirm that NAAG acts postsynaptically, and to determine if β-NAAG also acts postsynaptically. Two subthreshold stimuli (10 sec) were given to the MPP with interpulse intervals (IPIs) of 20 and 80 msec. Neither exposure to 100 M β-NAAG (N=5; p>0.05; paired t-test) nor exposure to 50 M NAAG (N=3; p>0.05; paired t-test) affected paired-pulse depression of granule cell EPSP slopes recorded during paired pulse paradigms (Figure 3D). If necessary, stimulus intensity was adjusted prior to the paired-pulse paradigms to ensure that the EPSP caused by the first 10 sec stimulus of each pair matched that measured during baseline recordings. We conclude from these data that both NAAG and β-NAAG do not act at a presynaptic site. As a result of our ethyl glutamate, β-NAAG, and paired-pulse data, and our knowledge that NAAG is a specific agonist at the mGluR3 receptor subtype (Wroblewska *et al.*, 1997), we speculated that β-NAAG might be acting as an antagonist of the action of NAAG at the mGluR3 receptor to reverse the effect of NAAG on LTP.

Figure 4 β-NAAG has no effect on IP3 formation in CHO cells expressing group I mGluRs. *A)* Increasing concentrations of β-NAAG (1-300 μM) had no effect on mGluR1α-mediated increases in IP3 formation when cells were stimulated with 15 μM glutamate (N=9; p>0.05; Student s t-test). *B)* Increasing concentrations of β-NAAG (1-300 μM) had no effect on mGluR5-mediated increases in IP3 formation when cells were stimulated with 5 μM glutamate (N=9; p>0.05; Student s t-test). The mGluR1α or mGluR5 subtypes were expressed in Chinese hamster ovary (CHO) cells. The cells were incubated for 60 min at 37°C in the presence of lithium (10 mM), and IP3 accumulation was measured as a percentage of maximal stimulation in response to glutamate. The mGluR1α affinity for glutamate is less than mGluR5 s. We therefore used two concentrations of glutamate to study the effects of β-NAAG on mGluR1α- and mGluR5-mediated increases in IP3 formation. These data represent means ± SEM, in triplicate.
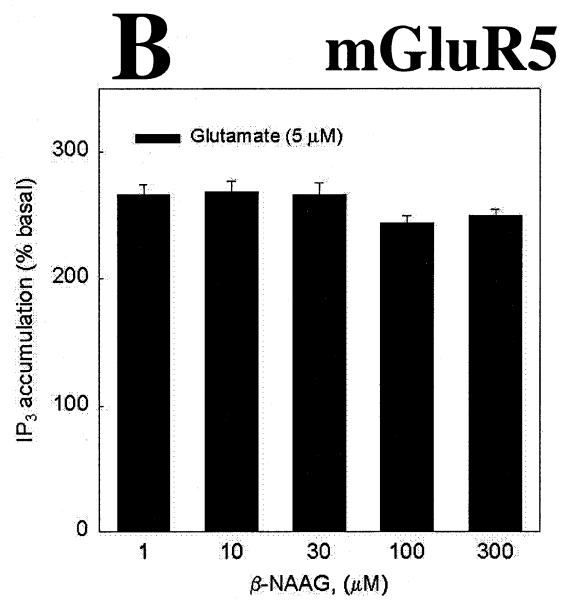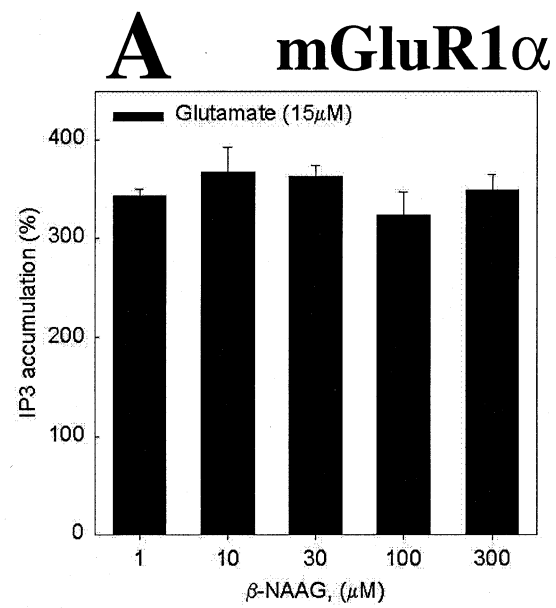
Figure 4

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β-NAAG affects group II but not group I or III mGluRs

We showed previously that NAAG does not activate group I mGluRs (Wroblewska *et al.*, 1997). Since β-NAAG reversed the effects of NAAG in our LTP experiments, we predicted that β-NAAG was acting as an antagonist to NAAG at the group II mGluR3 subtype. In addition, we predicted that β-NAAG would not affect either group I or group III mGluRs.

To rule out an effect of β-NAAG on the group I mGluRs, we used glutamate to stimulate IP3 formation in CHO cells stably expressing mGluR1α and mGluR5 in the presence of varying concentrations of β-NAAG. Since the affinity of mGluR1α for glutamate is less than that of mGluR5 (see (Conn and Pin, 1997) for review), we used 15 μM glutamate to activate the mGluR1α subtype and 5 μM glutamate to activate the mGluR5 subtype. Neither the group I mGluR subtype mGluR1α (Figure 4A), nor mGluR5 (Figure 4B) mediated increases in IP3 formation were changed in the presence of increasing concentrations of β-NAAG (1-300 μM; $N = 9$ $p>0.05$; Student s t-test). We conclude that β-NAAG does not affect group I mGluRs.

We tested the effects of β-NAAG on both group II and group III mGluRs. We used glutamate (groups II and III), *trans*-ACPD (group II), or AP4 (group III) to activate mGluR-mediated decreases in forskolin-stimulated cAMP. We found that β-NAAG (100 μM) blocked the ability of glutamate and *trans*-ACPD to decrease cAMP levels induced by forskolin. In contrast, β-NAAG failed to block the ability of AP4 to decrease cAMP *via* the group III mGluRs ($N=9$; $p>0.05$; Student s t-test; Figure 5A). These results show that β-NAAG has no effect on group III mGluRs. In addition, these data suggest that the

Figure 5. β-NAAG affects group II but not group III mGluRs. *A)* The nonspecific mGluR agonist glutamate (100 M), the group II agonist trans-ACPD (100 M), and the group III mGluR agonist L-AP4 (10 M) were used to test the effects of β-NAAG on forskolin-induced cAMP levels in cerebellar granule cells. β-NAAG blocked the effects of glutamate and trans-ACPD-stimulated group II mGluRs, but had no effect upon AP4-stimulated group III mGluRs (N=9; p>0.05; Student s t-test). *B)* DCG-IV (3 M) decreased cAMP in cerebellar granule cells to 55–10 percent of maximal forskolin-stimulated levels. β-NAAG (30 and 100 M) blocked this decrease (N=6; p<0.05; Student s t-test). The data (mean–SEM, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 M forskolin. Star represents significant difference from DCG-IV-treated cells in the absence of β-NAAG.
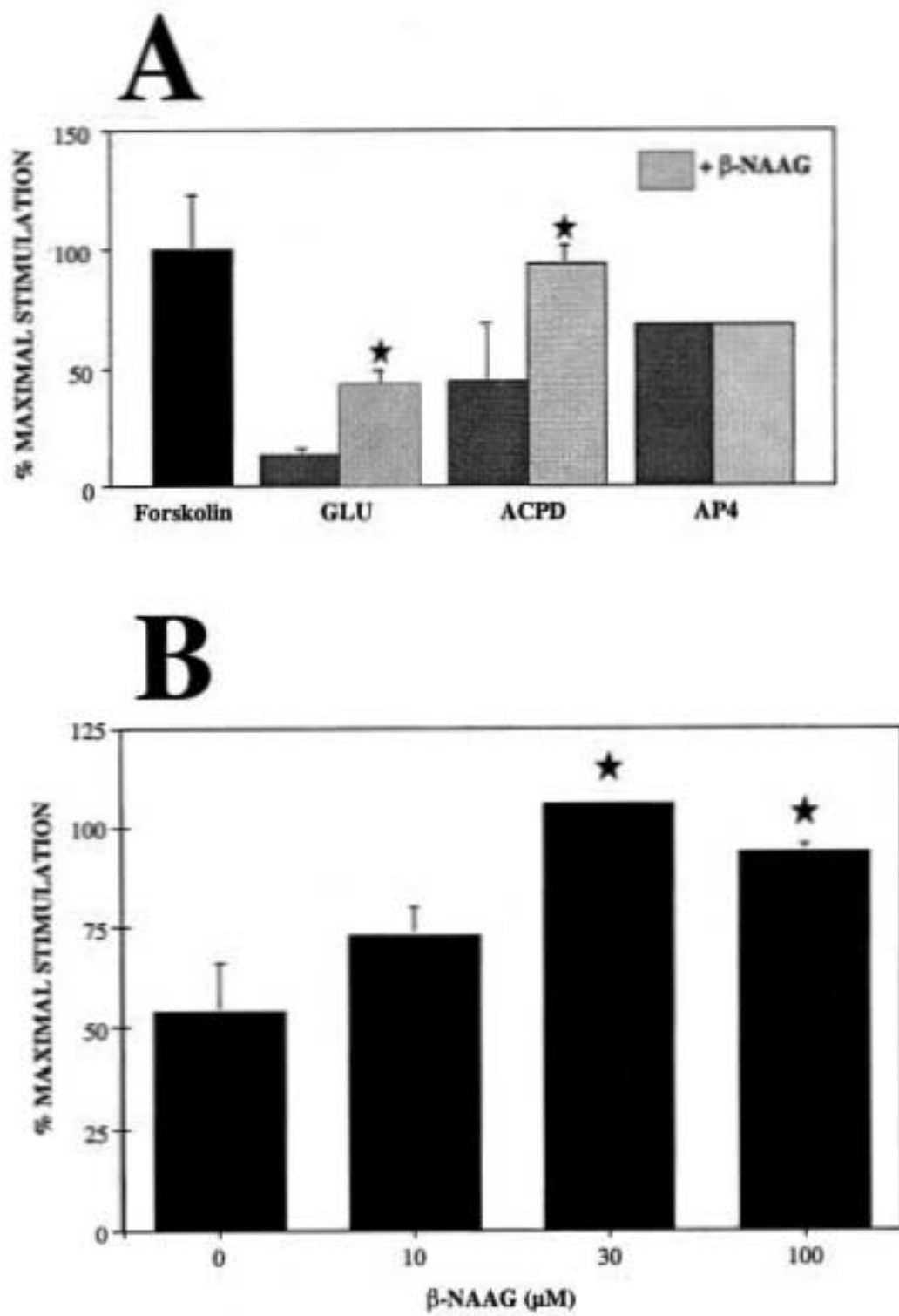
Figure 5

blockade of glutamate and *trans*-ACPD by β-NAAG is mediated through the group II mGluRs.

To verify the efficacy of β-NAAG against group II mGluRs, increasing concentrations of β-NAAG (10-100 μM) were applied to cerebellar granule cells that were treated with forskolin (10 μM) and DCG-IV (3 μM), an agonist for mGluR2 and mGluR3 (Figure 5A). Both 30 and 100 μM β-NAAG were found to block the action of DCG-IV in these neurons (Figure 5B; N=6; p<0.05; Student s t-test).

We conclude from these results that β-NAAG is an antagonist at the group II mGluRs, but not at the group I or group III mGluRs.

β-NAAG antagonizes mGluR3, but not mGluR2, receptors

We have shown previously that NAAG decreases forskolin-stimulated cAMP levels in cerebellar granule cells in culture (Wroblewska *et al.*, 1993). Moreover, using cell lines expressing single subtypes of metabotropic glutamate receptors, we have shown that NAAG selectively activates the group II metabotropic glutamate receptor subtype mGluR3 (Wroblewska *et al.*, 1997). To determine if one or both of the two group II mGluR subtypes (mGluR2 or mGluR3) is antagonized by β-NAAG, we tested for specificity using similar methods.

We tested the effects of β-NAAG on the mGluR2 receptor subtype stably transfected into a CHO-mGluR2 cell line. β-NAAG had no effect on either DCG-IV (Figure6A) or *trans*-ACPD (Figure 6B) mediated decreases in forskolin stimulated cAMP (N=5; p>0.05; Student s t-test). Therefore, we conclude that β-NAAG does not act as an antagonist at the mGluR2 receptor subtype.

Figure 6. β-NAAG has no effect on the mGluR2 receptor subtype. β-NAAG has no significant effect on DCG-IV (A) or *trans*-ACPD (B) mediated decreases in forskolin stimulated cyclic AMP formation in the CHO-mGluR2 cell line (N=5; p>0.05; Student s t-test). The cells were preincubated for 10 min in the medium (phosphate buffered saline, pH 7.4), and incubated for 7 min with forskolin (10 μM) and increasing concentrations of DCG-IV (0.1-30 μM, A) or *trans*-ACPD (0.1-300 μM, B) without (_), and with (_) β-NAAG (100 μM). The data (mean ± SEM, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 μM forskolin. Data were fit to a log dose-response curve using Prism 2.0 (GraphPad Software, Inc).

Figure 6

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To test the specificity of β-NAAG for the mGluR3 receptor subtype, we used cultured cerebellar granule cells which express group I, II and III mGluR subtypes. Cerebellar granule cells were stimulated with forskolin (10 μM) to increase cAMP levels. NAAG (1-300 μM) significantly inhibited the forskolin-stimulated increase in cAMP levels, while β-NAAG (100 μM) blocked the effect of all concentrations of NAAG (N=5; p<0.05; Student s t-test; Figure 7A). These data strongly suggest that β-NAAG is an antagonist at the mGluR3 receptor.

To test the ability of β-NAAG to block the effects of NAAG, we used forskolin to stimulate a BHK-mGluR3 cell line in the presence of increasing concentrations of NAAG (1-300 μM) with and without 100 μM β-NAAG (Figure 7B). NAAG significantly inhibited the forskolin-stimulated cAMP levels, while β-NAAG blocked the effect of NAAG (N=5; p<0.05; Student s t-test). We conclude that our data, obtained through the use of cultured cells expressing various mGluR receptor subtypes, demonstrate an action of β-NAAG as a specific antagonist at the mGluR3 receptor.

Figure 7. β-NAAG blocks NAAG inhibition of forskolin-stimulated cAMP formation *via* mGluR3. *A)* Rat cerebellar granule cells, which express mGluR3 and respond to 10 μM forskolin, were utilized to test the effects of increasing concentrations of NAAG (1-300 μM) on cAMP formation, in the absence or presence of 100 μM β-NAAG. NAAG decreased cAMP levels in a concentration-dependent manner (closed circles). 100 μM β-NAAG in the presence of NAAG blocked this decrease in cAMP levels (open circles; N=5; p<0.05; Student's t-test). The data (mean±SEM, N=5, in triplicate) are expressed as a percentage of the cAMP levels in the presence of 10 μM forskolin. Star represents significant difference from NAAG-treated cells (p<0.05). *B)* β-NAAG blocks the inhibitory effect of NAAG on forskolin-stimulated cAMP formation in the BHK-mGluR3 cell line (N=5; p<0.05; student's t-test). The cells were preincubated for 10 min in the medium (phosphate buffered saline, pH 7.4), and incubated for 7 min with forskolin (10 μM) and increasing concentrations of NAAG (1-300 μM) without (_), or with (_) β-NAAG (100 μM). The data (means ± SEM, n = 5, in triplicate) are expressed as a percentage of cAMP levels in the presence of 10 μM forskolin. Curves were fit to a log d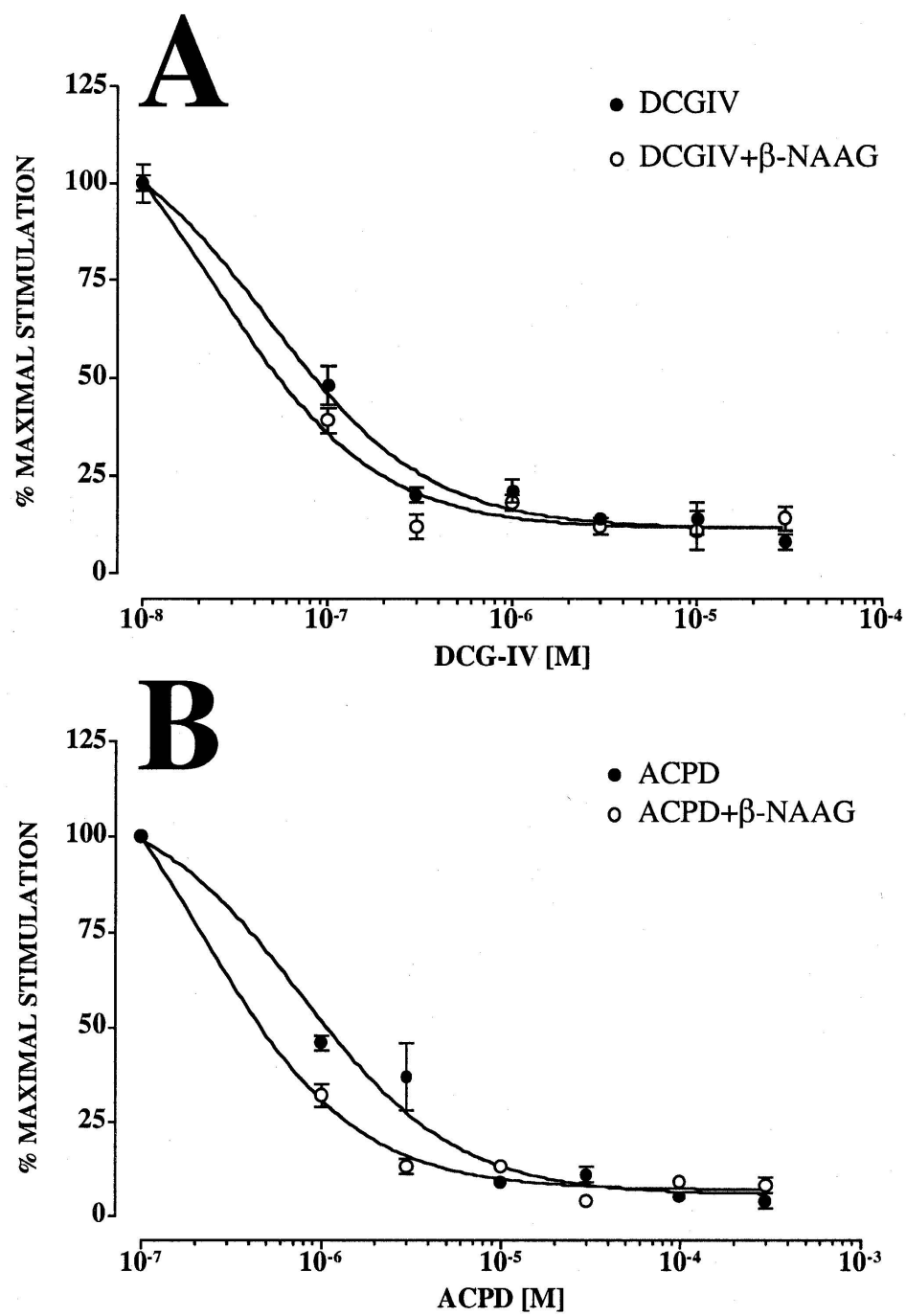ose-response curve using Prism 2.0 (GraphPad Software, Inc). ★ Significantly different from NAAG-treated cells with p<0.05.

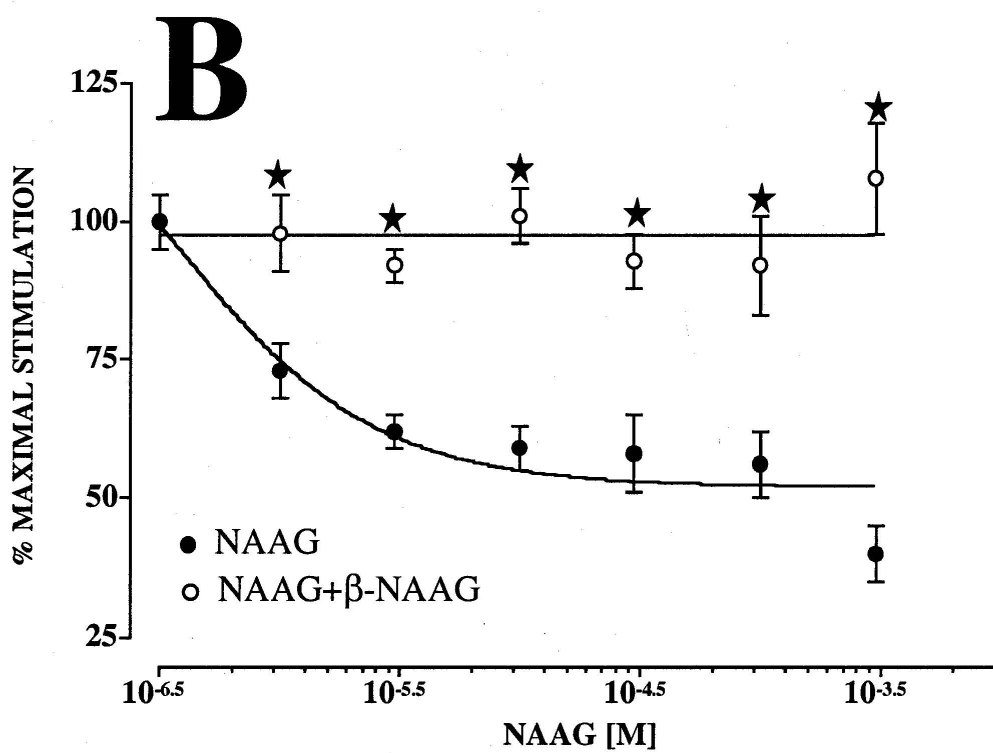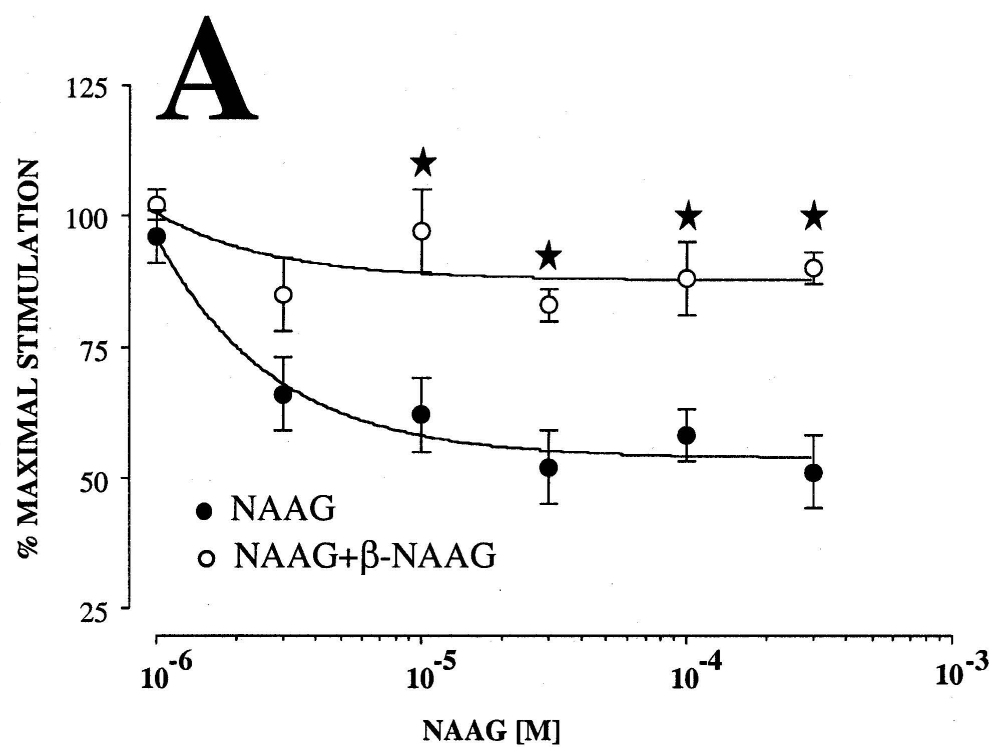Figure 7

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Discussion

This is the first study to identify β-NAAG as a specific antagonist of the mGluR3 receptor. It is also the first to identify the mGluR3 receptor as a target for NAAG in LTP. In this paper we show that 1) NAAG blocks LTP of extracellularly recorded EPSPs at the MPP-gc synapse, 2) NAAG does not affect NMDA receptor current in granule cells of the acute hippocampal slice, 3) β-NAAG and ethyl glutamate relieve the blockade of LTP by NAAG at the MPP-gc synapse, 4) β-NAAG alone does not affect MPP-gc EPSPs, 5) NAAG and β-NAAG do not affect paired-pulse depression of the MPP, 6) β-NAAG has no effect on group I or group III mGluRs, and 7) β-NAAG reverses mGluR3, but not mGluR2, mediated decreases in forskolin stimulated cAMP levels. These data demonstrate that mGluR3, linked to adenylyl cyclase, can block LTP at the MPP-gc synapse.

The peptide neurotransmitter, NAAG, is a selective agonist for mGluR3 in neurons (Wroblewska *et al.*, 1993; Wroblewska *et al.*, 1997) and glia (Wroblewska *et al.*, 1998). This receptor is coupled to a G protein that mediates a reduction in cytoplasmic levels of cAMP. We have reported the presence of NAAG in interneurons in the dentate gyrus as well as the presence of extracellular peptidase activity against NAAG in this tissue (Anderson *et al.*, 1986; Moffett *et al.*, 1993; Fuhrman *et al.*, 1994; Moffett and Namboodiri, 1995; Bzdega *et al.*, 1997). Additionally, group II receptors (mGluR2/3) have been demonstrated in neurons and glia in the dentate gyrus (Petralia *et al.*, 1996a). We previously observed that LTP in the dentate gyrus requires norepinephrine, acting on a β-adrenergic receptor, which stimulates adenylyl cyclase and produces an increase in cAMP (Stanton and Sarvey, 1985c). We therefore formulated the hypothesis that the

mGluR3 receptor could regulate the induction of LTP in this region of the hippocampus. Our results are consistent with this hypothesis.

We found that, similar to ethyl glutamate, β-NAAG antagonized the action of NAAG on LTP. This is consistent with a group II mGluR effect. The only previously reported action of β-NAAG was as a non-hydrolyzable inhibitor of nervous system GCP II activity (Serval *et al.*, 1990). In our preparation, GCP II was inhibited by the concentrations of phosphate and sulfate in the perfusion medium (Robinson *et al.*, 1987). Inhibition of GCP II should enhance rather than diminish the actions of NAAG. Because NAAG is a known agonist at the mGluR3 receptor, and β-NAAG reversed the effects of NAAG, we speculated that β-NAAG may act as a receptor antagonist.

In testing this, we found that β-NAAG did not affect glutamate stimulation of group I mGluRs expressed in CHO cells. Similarly, β-NAAG had no effect on group III mGluR stimulation with AP4. In contrast, β-NAAG antagonized the action of both NAAG and *trans*-ACPD on group II mGluRs (mGluR2 and mGluR3) in cerebellar granule cells. We concluded from these data that β-NAAG acts as a group II mGluR antagonist.

To differentiate between the two group II mGluR subtypes, we used cell lines transfected with either mGluR2 or mGluR3. Our results show that β-NAAG antagonizes the mGluR3 receptors but not mGluR2 receptors in transfected cells. These data support the conclusion that β-NAAG blocks the ability of NAAG to inhibit the induction of LTP in the medial perforant path of the dentate gyrus by acting as an antagonist of the mGluR3.

Beyond functioning as an mGluR3 agonist, NAAG clearly has been demonstrated to act as a low-potency agonist at NMDA receptors (Westbrook *et al.*, 1986; Trombley and Westbrook, 1990; Sekiguchi *et al.*, 1992)**.** In contrast, Sekiguchi *et al.* (Sekiguchi *et al.*, 1989) found that 5 M NAAG decreased the depolarization induced by both NMDA and quisqualate in oocytes that had been injected with rat brain mRNA. These results led to speculation that the partial inhibition of NMDA-induced transmitter release that was observed following 200 M NAAG application may be mediated by the peptide acting as an antagonist at this receptor (Puttfarcken *et al.*, 1993). Binding studies suggest that the peptide s affinity for rat brain NMDA receptors is 30-fold less than glutamate (Valivullah *et al.*, 1994). Given NAAG s low potency as an NMDA agonist in physiological studies, a potentially more parsimonious explanation of the findings of Sekiguchi *et al.* (Sekiguchi *et al.*, 1989), and Puttfarcken *et al.* (Puttfarcken *et al.*, 1993), is the possibility that the peptide may function as a partial agonist at this receptor. A second possibility is that the peptide interacts differentially with various subtypes of NMDA receptors expressed in the brain (Monyer *et al.*, 1994; Benke *et al.*, 1995; Wenzel *et al.*, 1995). A third possibility is that NAAG antagonizes synaptic release as an mGluR agonist, since other group II mGluR agonists have been shown to suppress synaptic release in several systems (Hayashi *et al.*, 1993; Ishida *et al.*, 1993; Schaffhauser *et al.*, 1998).

We have shown previously that MPP EPSP amplitude and area are decreased by NMDA receptor antagonists (Dahl *et al.*, 1990). Neither effect was seen during NAAG perfusion in our study. Additionally, we show that NAAG has no effect on NMDA

receptor currents in the granule cells of the acute hippocampal slice. These findings

support our hypothesis that NAAG is acting at the mGluR3 receptor.

An antibody that reacts with both mGluR2 and mGluR3 has been used to identify

receptors on both pre- and postsynaptic membranes (Testa *et al.*, 1994; Petralia *et al.*,

1996a; Shigemoto *et al.*, 1997). The activation of group II receptors decreases voltage-

dependent calcium currents (Sayer *et al.*, 1992; Chavis *et al.*, 1994; Bischofberger and

Schild, 1996) and synaptic release of neurotransmitter (Hayashi *et al.*, 1993; Ishida *et al.*,

1993; Schaffhauser *et al.*, 1998). In the present study, however, neither NAAG (50 or 200

M) alone, nor β-NAAG (100 M) alone, affected the EPSPs evoked by single or paired-

pulse stimulation of the MPP. Since the paired-pulse paradigm reflects the efficacy of

presynaptic transmitter release, these data support the conclusion that the peptide blocks

LTP by acting upon receptors located at sites distinct from the pre-synaptic nerve

terminal.

Our data support the hypothesis that mGluR3 receptor activation can suppress

LTP in the medial perforant path of the dentate gyrus. Neuromodulation in the MPP by

group II mGluRs is supported by the recent findings of Huang and colleaugues (Huang *et

al.*, 1999a) who report that, in the dentate gyrus in picrotoxin-treated, submerged slices

from young (40-80 g) rats, NAAG induces long-lasting depression similar to the group II

mGluR agonist DCG-IV. The absence of a detectable effect of NAAG on paired pulse

depression led to the conclusion that the peptide s action was on a post-synaptic site. Our

results confirm a postsynaptic site of action (i.e. the EPSP slopes at 20 and 80 ms IPIs,

recorded just prior to and after NAAG perfusion remained the same).

In addition to their findings above, Huang *et al.* (1999) reported that NAAG caused a significant increase in MPP EPSPs without altering presynaptic release. In contrast, NAAG had no effect on either EPSPs or presynaptic release in our experiments. One probable explanation is that differences in methodological approach (submerged vs. interface chambers, disinhibited vs. naturally inhibited slices, younger, 40-80 g vs. older, 80-210 g rats) may account for our contrasting findings. We show that NAAG blocks LTP. Given the presence of NAAG in hippocampal interneurons, these data raise the possibility that the endogenous peptide regulates synaptic plasticity. We have found that treatment with β-NAAG alone is not sufficient to generate long-lasting potentiation.

The relative contributions of endogenous glutamate and NAAG to the activation of this metabotropic receptor are unknown. While our discovery of β- NAAG's antagonist properties may permit detection of endogenous ligand activation of the mGluR3 receptor, discrimination between the actions of endogenous glutamate and NAAG at mGluR3 will be more complex. Nevertheless, our results are the first to show that 1) β- NAAG is an mGluR3 receptor antagonist and 2) the mGluR3 receptor functions to regulate activity-dependent synaptic potentiation in the hippocampus.

MODULATION OF EPILEPTIFORM BURST FREQUENCY AND DURATION BY THE

METABOTROPIC GLUTAMATE RECEPTOR SUBTYPE mGluR3

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Abstract

Perfusion of acute hippocampal slices with a solution containing an elevated concentration of potassium (10 mM) and a low concentration of calcium (0.5 mM) causes granule cells in the dentate gyrus to generate spontaneous epileptiform bursts and negative d.c. shifts in the extracellular potential [Schweitzer, Patrylo, Dudek. J.Neurophys 68:2016, 1992]. The contribution of the group II metabotropic glutamate receptors (mGluR2 and 3) to this burst activity remains unknown. We investigated modulation of spontaneous bursts in field potentials recorded from the granule cell layer and at the medial-perforant path-granule cell synapse. The absence of specific agonists or antagonists capable of differentiating between the mGluR2 or mGluR3 receptor subtypes led us to use N-acetyl-aspartyl-glutamate (NAAG) as a specific mGluR3 agonist and N-acetyl-β-aspartyl-glutamate (β-NAAG) as a specific mGluR3 antagonist. We investigated the mGluR3 receptor contribution to epileptiform activity. Our results show that the frequency of bursting in granule cells can be decreased by β-NAAG and the group II antagonist ethyl glutamate. The mGluR group II agonists bath applied after induction of spontaneous bursting had no effect on either burst frequency or duration. We conclude that 1) the group II mGluRs play a role in epileptiform bursting of granule cells, 2) the mGluR3 subtype mediates this modulation, and 3) the ability of mGluR3 subtype specific antagonists to decrease frequency of bursting may have therapeutic effects. *Supported by an Epilepsy Foundation Fellowship*.

Introduction

The metabotropic glutamate receptors (mGluR) can modulate induced epileptiform activity both in vivo and in vitro (see (Meldrum, 2000) for review). The mGluR family of G-protein linked receptors is divided into three groups based upon pharmacological, molecular, and functional properties (see (Conn and Pin, 1997; Anwyl, 1999; Schoepp *et al.*, 1999) for reviews). One particular model system used to study the role of mGluRs on induced epileptiform activity, is the acute hippocampal slice. Within this transverse section of the hippocampal formation various group I (mGluRs 1 and 5), II (mGluRs 2 and 3) and III (mGluRs 4a, 7a, 7b, and 8) subtypes of mGluRs are expressed (Testa *et al.*, 1994; Petralia *et al.*, 1996a). Interestingly, the distributions of the mGluR subtypes are region specific (Shigemoto *et al.*, 1997; Schaffhauser *et al.*, 1998). Therefore, various epileptiform-inducing agents can cause responses which will react differently to mGluR agonists and antagonists depending upon which regions of the hippocampal formation (CA1, 2, 3, hilus, dentate gyrus) are under investigation.

The group II mGluRs are of particular interest because of their localization to, and modulation of, the medial perforant pathway (MPP) of the adult rat dentate gyrus (Testa *et al.*, 1994; Macek *et al.*, 1996), respectively). MPP neuronal projections from the entorhinal cortex synapse upon the dendrites of granule cells in the midmolecular layer. Understanding the role that group II mGluRs have in modulation of dentate gyrus granule cells is important. These cells are typically resistant to epileptiform discharges as long as their inhibitory interneurons remain intact. Dentate gyrus granule cells provide a gate for neuronal activity to spread from glutamatergic and non-glutamatergic afferents into the hippocampus. Thus, the dentate gyrus can act as a control point for

epileptigenesis in the hippocampal formation as well as other downstream sites to which the hippocampus sends its efferent projections (Lothman *et al.*, 1992).

Perfusion of a high potassium low calcium solution causes granule cells in the dentate gyrus to generate spontaneous epileptiform bursts and negative d.c. shifts which are independent of NMDA, AMPA and $GABA_A$ receptors (Schweitzer *et al.*, 1992). The contribution of the metabotropic glutamate receptors (mGluRs) to this burst activity is unknown.

The goal of this study is to evaluate group II mGluR agonist/antagonist effects on epileptiform activity in dentate granule cells induced by perfusion of a high potassium low calcium solution. The effects of the mGluR3 specific agonist N-acetylaspartylglutamate (NAAG) and the mGluR3 antagonist β-NAAG on epileptiform activity are investigated.

Materials and Methods

*Preparation of hippocampal slices-*Male Sprague-Dawley rats (Taconic, Germantown, N.Y.) weighing 120-240 g were anesthetized with ketamine hydrochloride (100mg/kg i.p.) and decapitated. There was no detectable difference in responses due to the age of the rats. Transverse slices (400 m) of hippocampus were prepared using a McIlwain tissue chopper. Slices were placed in a modified Oslo interface recording chamber at 32-34¡ C and perfused at a rate of 3ml/min with artificial cerebrospinal fluid (ACSF) containing 26 mM $NaHCO_3$, 124 mM NaCl, 1.25 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4mM $CaCl_2$, and 10 mM dextrose; pH was adjusted to 7.4 by bubbling with a 95% CO_2/5 % O_2 gas mixture. Slices were allowed to equilibrate for at least two hours before recordings were initiated. Slices selected for study were perfused with an ACSF solution similar to the one above with the exception that 1.75 mM KCl, and 2.4mM $CaCl_2$ were replaced with 10 mM KCl and 0.5 mM. $CaCl_2$, respectively.

Electrophysiology- Figure 1A is a schematic of the hippocampal slice showing the placement of a 100 m diameter, monopolar, Teflon-insulated, stainless steel wire electrode, exposed only at the tip, in the medial perforant path (MPP) leading from the entorhinal cortex to the dentate gyrus. Standard glass micropipettes filled with 2M NaCl, 1-8 M‰ resistance, used for extracellular recordings, were positioned a minimum of 500 m from the stimulating electrode, and lowered to a final depth of 80 m into the slice. After equilibration, a stimulus intensity which allowed subthreshold stimulation at a 30 μs stimulus duration, and elicited a spike at a 40 μs stimulus duration, was selected. A half-maximum stimulus intensity was selected to confirm intact GABAergic inhibition.

Only slices showing complete abolition of the second spike at 20ms interpulse intervals in a paired-pulse paradigm were selected for study. Isolation of medial perforant path responses was confirmed using a previously reported method (McNaughton, 1980; Bramham *et al.*, 1997). Immediately following determination of intact inhibitory circuitry and electrode placement (Figure 1A) , we perfused the slices with ACSF containing high potassium (10 mM) and low calcium (0.5mM) (Schweitzer *et al.*, 1992). Test stimuli were delivered to the mid-molecular layer of the dentate gyrus every 30s to evoke a half-maximal reflected spike. After three hours, external stimuli were stopped, and spontaneous recordings were begun. Agonist and antagonist perfusion was started after recording a 20 minute baseline. Application of pharmacological agents was achieved by switching the chamber perfusion solution to a high potassium low calcium ACSF containing the drug. Drugs were perfused for a minimum of 30 minutes before recording the effects. The effects of washout were recorded 45 minutes after perfusing the high potassium low calcium ACSF without drug. Responses were amplified, filtered (d.c.-3 kHz), digitized at 20kHz (DAS-20 interface, Keithley Metrabyte, Taunton, MA) and stored for analysis using the pClamp 8 data acquisition analysis program (Axon Instruments, digidata). Significance was accepted at the $\alpha=0.05$ level.

Results

The mGluR3 agonists NAAG and DCG-IV have no effect on burst activity.

We confirm that in our hands a high K^+ low Ca^{2+} ACSF caused spontaneous bursting at the granule cell layer and medial perforant path-granule cell synapse. Stimulation during the three hours of perfusion of the high potassium (10 mM) low

Figure 1. The effects of ethyl glutamate and β-NAAG on nonsynaptic-epileptiform

activity. A) A schematic of the transverse hippocampal slice showing the placement of

the stimulating electrode in the medial perforant path (MPP) and the extracellular field

recording electrodes. Banners above the recording electrodes show characteristic

bursting patterns recorded at the MPP-granule cell synapses and granule cell bodies. B)

100 µM ethyl glutamate and 100 µM β-NAAG decrease burst frequency in granule cells

(N=3; $p<0.05$, ANOVA). Both antagonists were perfused after induction of spontaneous

bursting. Star represents significant difference from control ($p<0.05$).
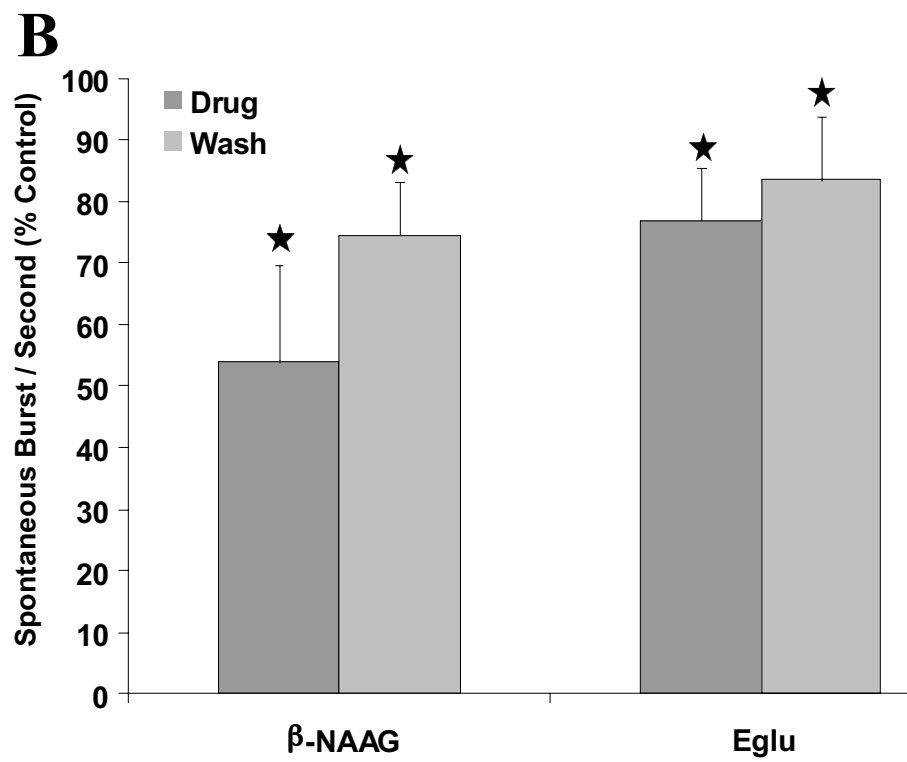
Figure 1

calcium (0.5mM) was necessary to initiate the spontaneous bursting. Without stimulation, the bursting pattern took longer to appear (approximately 4-6 hours) or did not appear at all (data not shown).

We found that perfusion of DCG-IV appeared to increase the susceptibility of the dentate granule cells and the MPP-gc synapse towards spreading depression after establishment of spontaneous activity making it difficult to test drug effects on spontaneous bursting (N=2; data not shown). When we tested the effects of the mGluR3 specific agonist NAAG, we found that NAAG had no effect on spontaneous bursting frequency (N=3; data not shown).

Group II mGluR antagonist ethylglutamate decreases burst frequency.

To further evaluate a possible modulatory role for the group II mGluRs, we used the group II mGluR specific antagonist ethyl glutamate (EGlu). We found that EGlu (100 μM) caused a decrease in the frequency of spontaneous bursting (Figure 1B, N=3; ANOVA). When EGlu was washed out of the slices, we found that the burst frequency increased but remained significantly lower than that found in control (Figure 1B, N=3, ANOVA). These results confirmed our prediction that the group II mGluRs can modulate epileptiform activity at the MPP-gc synapse and in the granule cells .

The mGluR3 specific antagonist β-NAAG decreases burst frequency.

To identify if the effect seen with EGlu was due to blocking the group II mGluR3 receptor subtype, we used the mGluR3 specific antagonist β-NAAG. We previously identified β-NAAG as a tool for differentiating between the mGluR2 and mGluR3

Figure 2. The effects of β-NAAG on nonsynaptic-epileptiform activity. 2A) A representative experiment showing a control recording of spontaneous bursting taken after at least three hours of high potassium low calcium ACSF. 2B) 200 μM β-NAAG, perfused after induction of spontaneous bursting, caused a temporary decrease in the frequency of bursting recorded at both the granule cell body layer and at the MPP-gc synapses. 2C) Washout of 200 μM β-NAAG with high potassium low calcium ACSF resulted in the return of the bursting frequency seen in control.

Figure 2

receptors (Lea *et al.*, 2001). We found that β-NAAG (200 μM) decreased the spontaneous bursting frequency at the MPP-gc synapse and in the granule cell (Figure 1B, N=3, ANOVA). When β-NAAG was washed out of the slices, we found that the burst frequency increased but remained significantly different from control (Figure 1B, N=3, ANOVA). Figure 2 shows representative traces recorded 1) in the presence of a high potassium low calcium ACSF without drugs (Figure 2A), 2) in which perfusion of 200 μM β-NAAG caused a decrease in the frequency of burst activity (Figure 2B), and 3) after washout of 200 μM β-NAAG (Figure 2C). These results confirm that the mGluR3 receptor is involved in the regulation of burst frequency at the MPP-gc synapse and in the granule cells.

Discussion

This is the first study to show that the group II mGluR antagonist EGlu and the mGluR3 specific antagonist β-NAAG decrease burst frequency in the granule cells of the rat dentate gyrus brought about by perfusion of a high potassium (10 mM) low calcium (0.5 mM) solution. In addition, we report that perfusion of the group II mGluR agonist DCG-IV and the mGluR3 specific agonist NAAG had no effect on burst frequency if perfused 20 minutes after spontaneous bursting began.

The induction of epileptiform activity in the granule cells of the dentate gyrus using high potassium and low calcium containing ACSF was characterized by Schweitzer et al. (1992). This epileptiform activity was termed nonsynaptic by Schweitzer and colleagues because their study showed that the NMDA receptor antagonist D,L-2-amino-5-phosphonopentanoate (AP-5), the $GABA_A$ receptor antagonist

bicuculline methiodide, and the AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), did not alter spontaneous epileptiform bursting in the dentate gyrus. Nevertheless, a possible modulatory role *via* the mGluR family remained unstudied, until now, despite findings that the mGluRs are involved in epilepsy (Sheardown, 1992; Burke and Hablitz, 1994; Arvanov *et al.*, 1995; Burke and Hablitz, 1995; Dalby and Thomsen, 1996; Ghauri *et al.*, 1996; Rutecki and Yang, 1997; Keele *et al.*, 1999; Wong *et al.*, 1999; Galoyan and Merlin, 2000; Klodzinska *et al.*, 2000).

Understanding the role that group II mGluRs have in modulation of dentate gyrus granule cells is important since these cells can gate neuronal activity spreading from glutamatergic and non-glutamatergic afferents into the hippocampus. The group II mGluRs modulate epileptiform activity in hippocampal CA3 neurons (Merlin *et al.*, 1995) and have been shown to modulate EPSPs recorded in the afferent projections to the granule cells of the hippocampus (Macek *et al.*, 1996; Huang *et al.*, 1997b). We found that the group II mGluR agonist DCG-IV caused spreading depression in rat hippocampal dentate granule cells. This finding was not a complete surprise to us. DCG-IV in normal ACSF causes a significant decrease in EPSPs recorded at the MPP-gc synapse within 10-20 minutes of perfusion in hippocampal slices with intact $GABA_A$ inhibition (our data, unpublished), and another using slices treated with picrotoxin (Huang *et al.*, 1997b). We found that the group II mGluR specific antagonist EGlu decreased burst frequency. These results support a role for group II mGluR modulation of nonsynaptic dependent epileptiform activity induced in the dentate gyrus of the rat hippocampus. We next investigated the contribution of the group II mGluR3 subtype.

The absence of generally accepted agonists or antagonists capable of differentiating between the mGluR2 or mGluR3 receptor subtypes, led us to use N-acetyl-aspartyl-glutamate (NAAG), an endogenous specific mGluR3 agonist (Wroblewska *et al.*, 1997), and the selective mGluR3 specific antagonist N-acetyl-β-aspartyl-glutamate (β-NAAG; (Lea *et al.*, 2001)) to look at the mGluR3 receptor contribution to epileptiform activity. The use of β-NAAG came about from earlier studies in this lab which showed that NAAG blockade of LTP in the dentate gyrus (Lea *et al.*, 2001) can be prevented by using β-NAAG . In a collaborative effort, we determined that the ability of β-NAAG to block NAAG was mediated through the mGluR3 and not the mGluR2 receptor subtype.

In this study, we found that NAAG had no affect on spontaneous activity in the hippocampal dentate gyrus granule cells. The mGluR3 receptor is known to be expressed by astrocytes (Condorelli *et al.*, 1997; Wroblewska, 1998; Schools and Kimelberg, 1999) and has been shown to upregulate the high-affinity glutamate transporter GLAST in astrocytes (Gegelashvili *et al.*, 2000). These results suggest that although levels of NAAG are increased in epileptic brains, the peptide will have no noticeable effect on epileptiform activity despite its ability to upregulate GLAST. Indeed, Merlin and colleagues (1995) suggests from their results studying picrotoxin treated, acute guinea pig hippocampal slice CA3 neurons, that the overall effect of glutamate stimulation of

various mGluRs during epileptic activity, is an increase in the frequency of synchronized discharges.

In contrast to our agonist data, β-NAAG decreased epileptiform bursting in granule cells of the dentate gyrus similar to EGlu. In support of these findings, Merlin and colleagues (1995) found that antagonists to mGluRs also decrease burst frequency in hippocampal CA3 neurons. We conclude from our study that, 1) group II mGluR antagonists can decrease nonsynaptic-epileptiform activity, 2) antagonists of the mGluR3 receptor subtype can decrease nonsynaptic-epileptiform activity, and 3) that the ability of group II mGluR, and specifically the mGluR3 subtype specific antagonists to decrease frequency of bursting may have potential therapeutic effects.

General Discussion

The focus of this dissertation was to determine if modulation of epileptiform activity and long-term potentiation (LTP) could be accomplished by stimulation and/or inhibition of the group II metabotropic glutamate receptors. In the two papers presented in this dissertation, I report that this is the first study to identify β-NAAG as a specific antagonist to the group II mGluR3 subtype and the first to identify the mGluR3 receptor as a target for NAAG in LTP. In addition, this is the first study to show that epileptiform bursting in granule cells of the hippocampal dentate gyrus is decreased by antagonists of group II mGluRs and by β-NAAG.

Summary of Findings

In the first paper of this dissertation, I am the first to show that 1) N-acetyl-L-aspartyl-L-glutamate (NAAG) blocks LTP of extracellularly recorded EPSPs at the MPP-gc synapse, 2) NAAG does not affect NMDA receptor current in granule cells of the acute hippocampal slice, 3) β-NAAG and ethyl glutamate relieve the blockade of LTP by NAAG at the MPP-gc synapse, 4) β-NAAG alone does not affect MPP-gc EPSPs, 5) NAAG and β-NAAG do not affect paired-pulse depression of the MPP, 6) β-NAAG has no effect on group I or group III mGluRs, and 7) β-NAAG reverses mGluR3, but not mGluR2, mediated decreases in forskolin stimulated cAMP levels. These data demonstrate that mGluR3, linked to adenylyl cyclase, can modulate LTP at the MPP-gc synapse and that the mGluR3 receptor is not a presynaptic receptor.

In the second paper I am the first to show that 1) the group II mGluR antagonist ethyl glutamate and the mGluR3 specific antagonist β-NAAG decrease burst frequency

in the granule cells of the rat dentate gyrus brought about by perfusion of a high

potassium (10 mM) low calcium (0.5 mM) solution, and 2) perfusion of the group II

mGluR agonist DCG-IV and the mGluR3 specific agonist NAAG had no effect on burst

frequency if perfused 20 minutes after spontaneous bursting began.

Significance

        Both the NMDA and group II mGluR receptors have been implicated in synaptic

plasticity (Harris *et al*., 1984; Huang *et al*., 1997a; Huang *et al*., 1997b; Huang *et al*.,

1999a; Huang *et al*., 1999b)) and epilepsy (Herron *et al*., 1985; Slater *et al*., 1985; Merlin

et al., 1995). Interestingly, levels of the group II mGluR3 subtype specific agonist NAAG

increase in KA (Koller *et al*., 1984) and kindling (Meyerhoff *et al*., 1985; Meyerhoff *et

al*., 1989; Meyerhoff *et al*., 1992c), models of epilepsy. Previous research on NAAG

supports a neuromodulatory role on excitatory neurotransmission *via* attenuation of

NMDA receptor activation, and decreased glutamate release from presynaptic

glutamatergic neurons caused by activation of the group II mGluR subtype mGluR3 (see

(Coyle, 1997) for review). These data suggest that NAAG may play a role in modulation

of synaptic plasticity and epileptiform activity.

        In the first part of this dissertation, I investigated the possible modulatory role of

NAAG on LTP in the MPP of the rat hippocampal dentate gyrus. The MPP afferents

synapse onto dendrites of granule cells of the hippocampal dentate gyrus forming MPP-

gc synapses. LTP of EPSPs recorded at the MPP-gc synapse requires the activation of

NMDA receptors (Harris and Cotman, 1986; Herron *et al*., 1986; Burgard *et al*., 1989), and increases in cAMP levels (Stanton and Sarvey, 1985c; Stanton and Sarvey, 1985b; Blitzer *et al*., 1995; Nguyen and Kandel, 1996; Blitzer *et al*., 1998). Macek and colleauges (Macek *et al*., 1996), showed that group II mGluRs are partially responsible for decreasing EPSPs in the midmolecular layer (MPP) of the dentate gyrus. Due to the lack of any specific antagonists to either mGluR2 or mGluR3, however, they were unable to identify which of the two group II mGluR subtypes was responsible for decreasing the EPSPs. These findings coupled with one showing NAAG distribution along the suprapyramidal blade of the dentate gyrus (Moffett and Namboodiri, 1995) suggested that NAAG may be able to modulate synaptic plasticity at the MPP-gc synapse and epileptiform activity in the granule cells of the dentate gyrus. I found that NAAG does block LTP in the MPP and that the NMDA receptor is not involved. In the process of investigating the role of NAAG in LTP, I became the first to discover β-NAAG as a specific antagonist to the mGluR3 receptor. This finding allowed me to conclude that NAAG blocks LTP via the mGluR3 receptor. In addition, I discovered that NAAG and β-NAAG had no effect on paired-pulse depression in the MPP. This finding was important because it indicates that the mGluR3 receptor is not a presynaptic receptor.

In the second part of this dissertation, I investigated the possible modulatory role of the group II mGluRs on epileptiform activity in the granule cells of the rat dentate gyrus. Understanding the role that group II mGluRs have in modulation of dentate gyrus

granule cells is important. These cells, typically resistant to epileptiform discharges, can provide a 'gate' for epileptic activity to spread from glutamatergic and non-glutamatergic afferents into the hippocampus once the extracellular potassium level rises and the calcium level decreases during epileptiform bursting. In addition to responding to changes in the extracellular ion concentration, these cells can exhibit epileptiform bursting if there is a decrease in the amount of inhibition provided by the GABA interneurons. Thus, the dentate gyrus can act as a control point for epileptigenesis in the hippocampal formation as well as other downstream sites to which the hippocampus sends its efferent projections (Lothman *et al*., 1992).

I initially began my study of epileptigenesis in the dentate gyrus of the hippocampal slice using kainic acid. It was previously reported that 10 μM kainic acid would induce epileptiform activity in the granule cells of the rat hippocampus (Westbrook and Lothman, 1983). If I could create a reproducible model for inducing epileptiform activity, a possible modulatory role for the group II mGluRs could be evaluated. I found that 10 μM kainic acid induced epileptiform activity in the granule cells in only a small percentage of my experiments (approximately 30%). I concluded that although kainic acid is successful in producing epileptic activity in areas CA1 and CA3 of the hippocampus, it is not a useful model to study epileptigenesis in the dentate gyrus. I therefore investigated other models known to induce epileptiform activity in the granule cells of the dentate gyrus.

Bicuculline is a known antagonist at the $GABA_A$ receptor. It is well known that another $GABA_A$ receptor antagonist, picrotoxin, is used in many labs studying LTP to

disinhibit the granule cells in the dentate gyrus. This experimental protocol is used primarily due to the inability of many labs to induce LTP in the granule cells without removing endogenous inhibition within the dentate gyrus. Preliminary experiments showed that bicuculline was indeed successful in inducing multiple spikes in the hippocampus using subthreshold stimuli. To characterize this epileptiform activity, I used AP5, a potent antagonist at the NMDA receptor. I found that 1) AP5 blocked the majority of the bursting activity caused by subthreshold stimuli given to the MPP in the presence of bicuculline, and that 2) group II mGluR agonists did not have an effect on the burst activity which remained after perfusion of AP5. Additional experiments in the lab also indicated that the burst activity which remained after AP5 perfusion was not abolished by an AMPA receptor antagonist. These findings, in addition to the fact that around this same time NAAG was shown to affect the release of GABA (Zhao *et al.*, 2001) led me to the decision to stop this series of experiments and to look into another model.

Perfusion of ACSF containing low calcium has also been shown to induce epileptiform activity in the granule cells of the hippocampal dentate gyrus. Preliminary experiments showed, however, that AP5 completely abolished the multiple spikes obtained by giving subthreshold stimuli in the presence of ACSF containing 0.5 mM calcium. These results led to the final model studied for inducing epileptiform activity in the granule cells.

Perfusion of a high potassium low calcium solution causes granule cells in the dentate gyrus to generate spontaneous epileptiform bursts and negative d.c. shifts which are independent of NMDA, AMPA and $GABA_A$ receptors (Schweitzer *et al.*, 1992). The

contribution of the metabotropic glutamate receptors (mGluRs) to this burst activity is unknown. I found that the group II mGluR agonist DCG-IV and the mGluR3 agonist NAAG did not affect epileptiform bursting in the granule cells if perfused after spontaneous activity began. In addition, I found that the group II mGluR antagonist ethyl glutamate and the mGluR3 antagonist β-NAAG decreased this epileptiform activity. I conclude from our study that the mGluR3 receptor subtype can modulate epileptiform activity, and that the ability of mGluR3 subtype specific antagonists to decrease frequency of bursting may have potential therapeutic effects.

Future Directions

The mechanisms induced by mGluR3 receptor activation warrant further investigation. Multiple models predict that it is involved in plasticity of the synapse. For example, the mGluR3 receptor is upregulated in stage 5 amygdala kindling and is believed to contribute to the development of long-lasting plastic changes associated with seizure activity (Al-Ghoul *et al.*, 1998). The mGluR3 receptor is also believed to be instrumental in plastic changes within the spinal cord during the development of hyperalgesia and pain (Boxall *et al.*, 1998). The role of the mGluR3 receptor in plastic changes which occur during pain was supported when agonists to the group II mGluRs dose-dependently enhanced formalin-induced nociception (Fisher and Coderre, 1996).
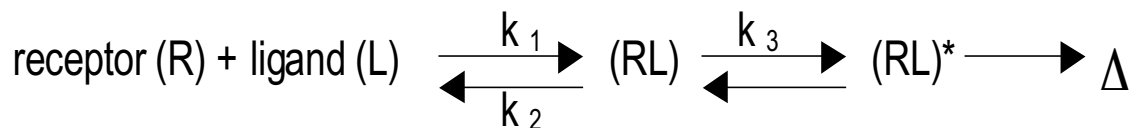
The mGluR3 receptor is known to be expressed by astrocytes (Condorelli *et al.*, 1997; Wroblewska, 1998; Schools and Kimelberg, 1999) and has been shown to upregulate the high-affinity glutamate transporter GLAST in astrocytes (Gegelashvili *et al.*, 2000). In addition, the mGluR3 receptor has been shown to decrease the release of GABA (Zhao *et al.*, 2001).

Previous findings predict that increasing GABA release by blocking the mGluR3 receptor would not affect the type of epileptiform bursting described in our second paper (Schweitzer *et al.*, 1992). This prediction however does not take into account the $GABA_B$ receptor. Further studies require the use of $GABA_B$ receptor agonists and antagonists. Increasing GABA release by blocking the mGluR3 receptor and activation of the $GABA_B$ receptor may be responsible for the inhibition of NAAG s ability to block LTP. It has already been shown that $GABA_B$ receptor activation causes a long-lasting potentiation in the dentate gyrus (Burgard and Sarvey, 1991). Again, further studies require the use of $GABA_B$ receptor agonists and antagonists.

Perhaps inhibition of the mGluR3 receptor on the astrocytes, and therefore decreased upregulation of GLAST, leads to an increased glutamate concentration in the extracellular space and perhaps this increase in extracellular glutamate effectively increases the extracellular osmolarity. Increased osmolarity has already been shown to decrease epileptiform activity (Roper *et al.*, 1993). Another possibility is that inhibition of the mGluR3 receptor on the astrocytes can lead to sustained levels of glutamate in the extracellular space. Sustained concentrations of glutamate in the extracellular space have already been shown to decrease the synchronous oscillations of intracellular calcium concentration shown to be required for repetitive excitation of some postsynaptic glutamatergic hippocampal neurons (Verderio *et al.*, 1999). These predictions support the conclusion that further studies need to be performed in order to elucidate the mechanisms by which the mGluR3 receptor mediates its effects.

Appendix A

I propose that by pharmacological definition, NAAG should be reported as a partial agonist at the NMDA receptor.

$$\text{receptor (R) + ligand (L)} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{(RL)} \overset{k_3}{\rightleftharpoons} \text{(RL)}^* \longrightarrow \Delta$$

In general, when receptor (R) is bound by ligand (L), it takes a given amount of time for a receptor-ligand (RL) complex to form. The rate at which this formation occurs is k_1; a second order rate constant (A + B → C; (Gilbert, 1992). The RL complex, once formed, can dissociate back to R and L alone, measured by k_2; a first order rate constant (A → B; (Gilbert, 1992), or can undergo a change which can be designated (RL)*. The rate at which the RL complex changes to (RL)* is k_3; another first order rate constant. From (RL)* formation, the ligand can modulate one to several effects (Δ).

An agonist in general is a ligand which upon binding to receptor can cause the reaction to proceed such that the first order rate constant k_3 is very high. That is, the agonist has high efficacy. A partial agonist has a k_3 usually fairly low or an efficacy greater than zero, but less than that of the full agonist. Partial agonists, depending upon the experimental parameters, may have agonist or antagonist properties (Gilman, 1980). For example, Puttfarken et al (1993) show that NMDA and L-Glu in the presence of NAAG are unable to induce the same [³H]-norepinephrine release that NMDA and L-Glu alone can produce. Indicating that NAAG may be an antagonist at the NMDA receptor. However, their dose-response plots indicate that the EC50 values are similar with or without the 100 M NAAG. This is a good indicator that the shift in response is due to a

partial agonist binding to the same receptor. Finally, the primary difference between agonist and antagonist is that the k_3 of an antagonist is equal to zero (Gilman, 1980) and therefore no ligand-evoked response should occur. This however is not the case with NAAG at the NMDA receptor.

Many of the papers which discuss NAAG seem at first to implicate a functional role as an antagonist at the NMDA receptor. Further analysis of their results, however, show that NAAG only partially antagonizes the effects of other substrates (agonists or antagonists). For example, 50 M NAAG has been shown to antagonize 25 M NMDA stimulated [^{3}H]-norepinephrine release in hippocampal slices by a maximum of 25% (IC50=5 M, (Puttfarcken *et al.*, 1993) . Valivullah et al (1994) showed that NAAG (IC50=8.8 M) was approximately 25-fold less potent as a competitor than glutamate (IC50=0.37 M) in the displacement of CGS-19755 from the NMDA receptor.

In addition to competing with NMDA receptor agonist or antagonist binding, NAAG-evoked effects, which can be blocked by specific NMDA receptor antagonists, implicate a partial agonist action of NAAG at the NMDA receptor. For example, NAAG-evoked currents in cultured mouse spinal cord neurons, cultured chick cerebellar neurons, and *Xenopus lavis* oocytes expressing NMDAR1 receptors (Westbrook *et al.*, 1986; Mori-Okamoto *et al.*, 1987; Sekiguchi *et al.*, 1992), respectively), NAAG-evoked excitation in rat lateral septal nucleus neurons (Joels *et al.*, 1987), and NAAG (500 M) stimulated [^{3}H]-norepinephrine release from hippocampal slices have been partially to completely blocked by the NMDA receptor antagonist , DL-2-amino-5-phosphonovaleric acid (APV). Other antagonists shown to compete with NAAG for receptor binding include MK801 (Pai and Ravindranath, 1991; Puttfarcken *et al.*, 1993)),

100 M (–)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (Puttfarcken *et al.*, 1993), 1000 M D- α-aminoadipate (DAA), 1000 M 2-amino-4-phosphonobutyrate (APB), and 6000 M glutamic acid diethyl ester (GDEE). The latter three, antagonized the depolarizing actions of NAAG by about 88%, 46% and 73%, respectively (Mori-Okamoto *et al.*, 1987).

Additional studies supporting the role of NAAG (1-1000 M) as a partial agonist of the NMDA receptor show dose-dependent suppression of climbing fiber responses in purkinje cell dendrites in cerebellar slices, and depolarization in *X. laevis* oocytes injected with guinea pig cerebellum mRNA (Sekiguchi *et al.*, 1989). When iontophoretically applied to cultured rat pup (12-day) suprachasmatic nucleus neurons (SCN), NAAG directly increased firing rates, and potentiated glutamate responses, in silent, regular and irregular SCN neurons (100,000 M NAAG; 20-100nA) (Bos and Mirmiran, 1993).

Since the results of all of the papers above show that NAAG evokes a response at the NMDA receptor, the first rate constant k3 for NAAG at the NMDA receptor must be greater than zero and therefore does not meet the criteria for an NMDA receptor antagonist. I therefore conclude, that the reference of Grunze and colleagues (Grunze *et al.*, 1996) to NAAG s ability to antagonize alveus stimulated LTP by acting as an endogenous NMDA receptor antagonist can more appropriately be described as a partial agonist effect of NAAG upon the NMDA receptor.

Probably one of the most critical experiments which needs to be performed is a pharmacological study investigating dose-response relationships taking into account NMDA and mGluR3 receptor activation by NAAG. With the use of specific NMDA and group II metabotropic receptor antagonists, as well as β-NAAG to distinguish between

mGluR2 and mGluR3 receptors, the contribution each receptor may have to a given

response could be evaluated.

Appendix B

The mGluRs affect Calcium Channels

To date, the mGluRs have been found to inhibit N-type calcium channels via a pertussis toxin-sensitive G-protein in multiple systems (*Neocortex*: (Swartz *et al.*, 1993; Choi and Lovinger, 1996; Stefani *et al.*, 1998a); *Hippocampus*: (Swartz and Bean, 1992; Sahara and Westbrook, 1993; Swartz *et al.*, 1993); *Striatum*: (Stefani *et al.*, 1994). The mGluRs inhibit L-type calcium channels in Neocortex (Sayer *et al.*, 1992), and cerebellar granule cells (Chavis *et al.*, 1994; Chavis *et al.*, 1995a) *via* an unidentified diffusible intracellular messenger (Anwyl, 1999). The effects do not seem to be due to PKC, high cAMP, PKA, tyrosine kinase or okadaic acid inhibition of phosphatase (Chavis *et al.*, 1995a; Chavis *et al.*, 1996; Chavis *et al.*, 1998; Shen and Slaughter, 1998). Lastly, P/Q-type calcium channels in Neorcortex (Stefani *et al.*, 1998a), and trapezoid body (Takahashi *et al.*, 1996) that are sensitive to ω-Agatoxin IVA are blocked by group I and II mGluR agonists. The mGluR agonists trans-ACPD (gp I and II mGluRs), (1s,3R)-ACPD (gp I and II mGluRs), quisqualate (gp I mGluRs, AMPA), L-AP4 (gp III mGluRs), and MCPG (gp 1 mGluRs) have also been implicated in the potentiation of voltage-gated Ca^{2+} channels (Rothe *et al.*, 1994; Chavis *et al.*, 1995a; Chavis *et al.*, 1995b; Chavis *et al.*, 1996; Chavis *et al.*, 1998).

The mGluRs affect Potassium Channels

Potassium channels are a major target for the mGluRs. Located on pre- and post-synaptic neurons as well as astrocytes, K^+ channels modulate neuronal excitability. In general, various K^+ channels are activated or inhibited by mGluRs. In cultured

cerebellar Purkinje cells (Netzeband *et al.*, 1997), the hippocampus (Charpak *et al.*, 1990; Charpak and Gahwiler, 1991; Miles and Poncer, 1993; Gereau and Conn, 1994; Davies *et al.*, 1995; Gereau and Conn, 1995a; Gereau and Conn, 1995b; Guerineau *et al.*, 1997) and dentate gyrus (Baskys and Malenka, 1991; Abdul-Ghani *et al.*, 1996a), and the basolateral amygdala (Womble and Moises, 1994), group I mGluRs block the tail current following depolarizing step voltages known as the slow Ca^{2+}-dependent afterhyperpolarizing K^+ current (I_{AHP}) *via* a G-protein dependent mechanism. This effect can be blocked by RGS4, a regulator protein of G-protein signaling (Saugstad *et al.*, 1998), enhanced by GTP-γ-S, reduced by GDP-β-S (Abdul-Ghani *et al.*, 1996b), and is PTX-insensitive (Gerber *et al.*, 1993). The mGluR I induced blockade of I_{AHP} was reduced by the protein tyrosine kinase inhibitors genistein, lavendustin-A and tyrophostin-B42. Intracellular Ca^{2+} does not seem to be a requirement for this blockade. IP_3R antagonists reduced the action of ACPD in the dentate gyrus implicating the possible involvement of PLC activation in I_{AHP} blockade. In contrast, staurosporine, chelerythrine or PKC19-31 and Walsh peptide did not affect I_{AHP} indicating that PKC and PKA may not be involved. Although, PKC activators phorbol 12,13-diacetate and phorbol 12-13-dibutyrate inhibited the I_{AHP}, the effects are postulated to be due to desensitization effects on G-protein coupling of the mGluR (Anwyl, 1999). Group II and III mGluRs did not block AHP (Gereau and Conn, 1995a). In addition to the blockade of I_{AHP}, group I mGluRs block the voltage-dependent slowly inactivating current (I_M), in the

nucleus tractus solitarius (Glaum and Miller, 1992), the hippocampus (Charpak *et al*., 1990; Charpak and Gahwiler, 1991; Shirasaki *et al*., 1994; Harata *et al*., 1996) and the basolateral amygdaloid nucleus (Womble and Moises, 1994).

The leak potassium current ($I_{K(leak)}$), in the geniculate thalamic relay neurons (McCormick and von Krosigk, 1992), CA3 neurons (Baskys and Malenka, 1991; Guerineau *et al.*, 1994; Haruta *et al.*, 1994), and the large caudate putamen cholinergic neurons (Takeshita *et al.*, 1996) was decreased by (1S,3R)-ACPD. This effect was blocked by GDP-β-S, and MCPG, and potentiated by GTP. Thus, indicating the requirement of a G-protein (Guerineau *et al.*, 1994).

Alteration in spike firing in certain neurons has been found to be mediated through blockade of I_{AHP}, $I_{K(leak)}$ and I_M currents. Agonists of the group I mGluRs have been shown to shift the firing mode of layer V neocortical (Wang and McCormick, 1993) and lateral geniculate thalamic (McCormick and von Krosigk, 1992) neurons from burst to single spike mode by inducing cell depolarization.

The inhibition of a basal and muscarinic m2-evoked inward rectifying channel (K_{ir} a.k.a. *GIRK*) in Xenopus oocytes expressing GIRK1 and 2 was found to be mediated via a PTX-insensitive G-protein and a bis-indolylmaleimide and staurosporine-sensitive PKC subspecies (Sharon *et al.*, 1997). It is of interest to note, however, that K_{ir} in cerebellar neurons is activated by group II mGluR agonists (Knoflach and Kemp, 1998). The decay of a slow Ca^{2+}-independent current ($I_{K(slow)}$) identified in hippocampal neurons of area CA3 is not blocked by typical K^+ channel blockers (egs. 4-AP, TEA, dendrotoxin, Ba, charybdotoxin, and glibenclamide), but is reduced by a high concentration of (1S, 3R)-ACPD (Luthi *et al.*, 1997).

The group I mGluR-induced slow Ca^{2+}-dependent current $(I_{K(Ca, slow)})$ recorded from pyramidal cells of the olfactory cortex and layer V of the neocortex was dependent upon Ca^{2+} entry, inhibited TEA and TBA, and was insensitive to external Ba^{2+}, Cs^+, 4-AP, and TTX (Constanti and Bagetta, 1991).

Activation of small (10 pS), intermediate (18-50 pS) and large (100-250 pS) conductance K^+ currents has been shown in neonatal hippocampal neurons in slices (Premkumar and Chung, 1995), in dissociated hippocampal neurons (Haruta *et al.*, 1994; Shirasaki *et al.*, 1994) , and cerebellar granule cells (Chavis *et al.*, 1996; Chavis *et al.*, 1998).

The mGluRs affect AMPA and NMDA Receptor mediated EPSCs

The AMPA- and NMDA-mediated responses can be separated from one another using either CNQX or NBQX to block the AMPA component, or AP-5 to block the NMDA component. O'Connor and colleagues (O'Connor *et al.*, 1995) used this technique to show mGluR-agonist induced long lasting potentiation of both EPSC responses in patched dentate granule cells. Ugolini and colleagues (Ugolini *et al.*, 1997) showed potentiation of both AMPA- and NMDA-induced potential changes in the ventral roots of 4-10 day old rat pup spinal cords. This response was blocked with the group I antagonists (S)-4-carboxy-phenyl-glycine (4CPG) or (+)-a-methyl-4-carboxyphenylglycine (MCPG) and the PKC blockers staurosporine or chelerythrine chloride. The group I mGluRs have been shown to reversibly potentiate this NMDA-mediated current in multiple cell types (*CA1:* (Aniksztejn *et al.*, 1991; Behnisch and Reymann, 1993; Harvey *et al.*, 1993; Fitzjohn *et al.*, 1996; Mannaioni *et al.*, 1996; Cohen

et al., 1999); *Purkinje cells*: (Kinney and Slater, 1992); *spinal cord motorneurons:* (Ugolini *et al.*, 1997); *principle striatal cells:* (Pisani *et al.*, 1997a) and *Xenopus oocytes:* (Kelso *et al.*, 1992). Group II and III mGluRs have no effect (Fitzjohn *et al.*, 1996; Pisani *et al.*, 1997a). Blockade of PKC-mediated phosphorylation of the NMDA receptor with sphingosine and the PKC inhibitory peptide PKCI (Aniksztejn *et al.*, 1991), staurosporine (Kelso *et al.*, 1992), staurosporine and calphostin C (Pisani *et al.*, 1997b), prevented the enhancement of NMDA-mediated current. Phorbol esters, activators of PKC, potentiated the NMDA-mediated current (*CA1*: (Aniksztejn *et al.*, 1991); *Xenopus oocytes*: (Kelso *et al.*, 1992); and *striatal cells*: (Pisani *et al.*, 1997b)).

The mGluRs modulate GABA release

The mGluRs can modulate GABA interneurons in two ways. First, group II or group III mGluRs, located at the axonal terminals, can inhibit GABA release (Poncer *et al.*, 1995; Schrader and Tasker, 1997); respectively). Inhibition of release is typically due to increased outward current (K^+) or decreased inward current (Na^+, Ca^{2+}) or a change in Cl^- conductance.   Second, group I mGluRs, located at the cell body of the interneurons, can stimulate the release of GABA (Miles and Poncer, 1993; McBain *et al.*, 1994; Jouvenceau *et al.*, 1995; Llano and Marty, 1995; Poncer *et al.*, 1995; Jonas *et al.*, 1998) by increasing inward current, causing depolarization of the cell, leading to increased action potentials and GABA release.

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